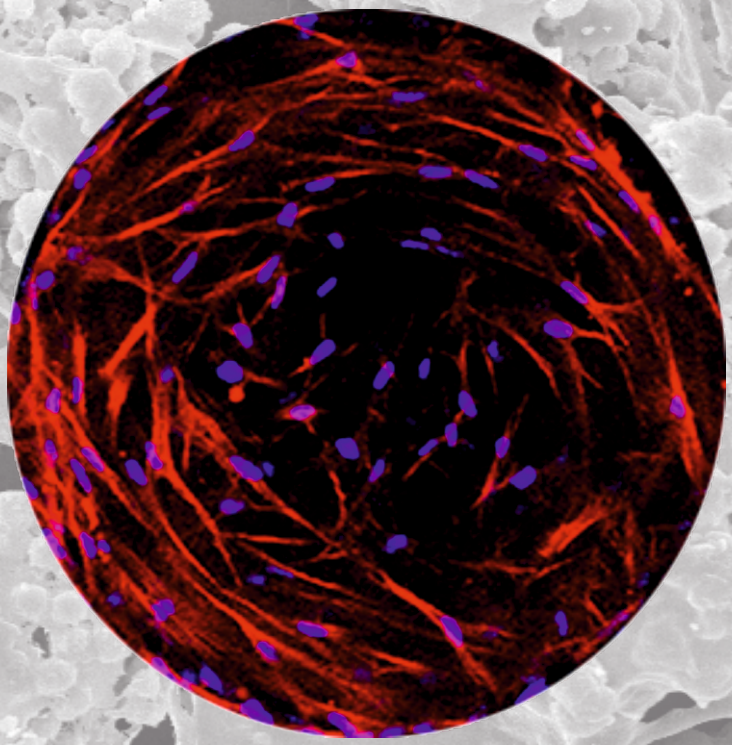

CELLULAR AND MOLECULAR ASPECTS OF CELL-CELL AND CELL-BIOMATERIAL INTERACTION

ASTGHIK HAYRAPETYAN

Cellular and Molecular Aspects of Cell-Cell and Cell-Biomaterial Interactions

Astghik Hayrapetyan

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This thesis is dedicated
to my beloved family.

CONTENTS

CHAPTER 1

Cellular & Molecular Aspects of Bone Regeneration

1.1 Bone

Bone is a rigid organ, constituting the human skeleton that supports and protects various organs of the body to maintain their function as a universal system. Bone tissue is connective tissue, which is composed of mineralized extracellular matrix in which different cell types, i.e. osteoblasts, osteoclasts, and osteocytes, are embedded for reconstruction, remodeling, resorption and mineralization processes of bone tissue. Bone defects can be caused due to various reasons, e.g. trauma, skeletal abnormalities, tumor resection and congenital disorders. In response to injury, bone has an intrinsic ability for regeneration, remodeling and repair. Although bone possesses these remarkable self-healing and regeneration capacities, in many clinical cases the regeneration of bone defects, especially those that exceed the critical size dimensions, this self-healing potential is insufficient to completely heal the defect. Consequently, critical size bone defects cannot be healed without surgical intervention [1] and represent a clinical challenge for surgeons that

is related to finding an appropriate method for bone defect healing [2]. Currently, the general treatment for bone defects is the use of autologous bone grafts (from the patient) or allograft (from a donor), the former which has become established as the "gold standard" in bone reconstructive surgery. However, these approaches have many limitations, including size limitation of available tissue, second surgery, donor site morbidity, risk of rejection and disease transmission (latter only when using allograft). Alternative treatment options for bone grafting are the use of alloplastic or allogenic materials, either or not in combination with adult stem cells (mesenchymal stromal cells [MSCs]) and/or signaling molecules (e.g. growth factors) [3]. In view of the promises of tissue engineering, regenerative medicine and adult stem cell technology, bone regenerative research is heavily focused on strategies that combine scaffolds, cells and signaling molecules. To achieve an advance level in tissue engineering strategies, it is important to understand the influence of scaffold properties (stiffness, topography), signaling factors (hormones, growth factors), metabolic (oxygen, Ca^{2+}) and inflammatory factors on cellular behaviour and osteogenic differentiation (Figure 1.1). This thesis is mainly focused on cell-cell and cell-biomaterial based aspects of bone regeneration involving adult stem cell-based approaches for (future) application in bone regenerative research and therapy.

1.2 Cell-based strategies in bone regenerative approaches

Among various cell types used in tissue engineering approaches, mesenchymal stromal cells (MSCs) have received major attention, because of their proliferation and multipotent differentiation potential, including differentiation into the osteogenic lineage. MSCs can be harvested from various tissues, including adipose tissue (AT), bone marrow (BM), cord blood, dental pulp,

skin, and placenta. However, the yield of MSCs isolated from these diverse tissues is different [5]. In view of this and because of apparent osteogenic potential, BM-MSCs and AT-MSCs are the most frequently used adult stem cells in bone regeneration research [6, 7, 8]. Still, the easy accessibility, low morbidity and relatively high yield make AT the most logical choice as the source for MSCs compared to other tissue sources [6, 9].

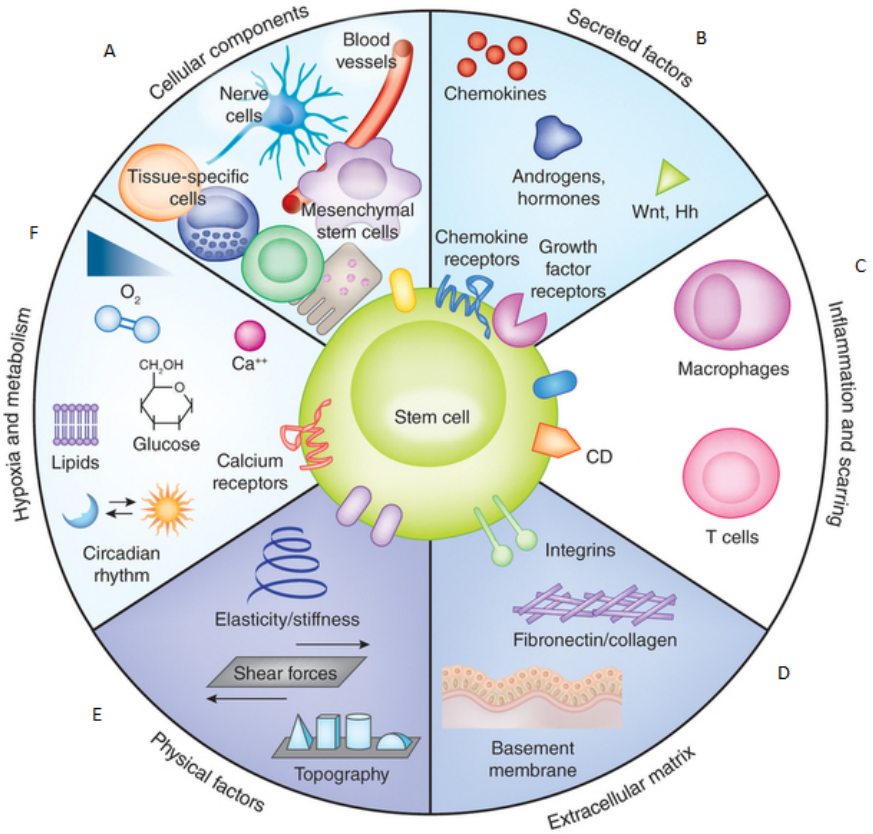

Figure 1.1: Factors involved in mesenchymal stromal cell differentiation [4]. **A**. Cellular components and cell-cell interaction, **B**. Secreted signaling factors and signal transduction, **C**. Inflammation and influence on osteogenic differentiation, **D**. Extracellular matrix formation, **E**. Influence of physical factors, **F**. Metabolic factors.

1.3 Cell-cell interaction & crosstalk

To develop cell-based bone regenerative strategies, it is necessary to understand natural bone regeneration, remodeling and fracture healing processes. All of these are complex physiological processes, involving multiple cell types (e.g. MSCs, angiogenic cells, immune cells) and multiple factors (acting through different signaling pathways), which coordinate cell migration, proliferation, osteogenic differentiation and extracellular matrix formation [10]. Bone is a highly vascularized tissue and vascularization is an essential process during bone fracture healing and regeneration (Figure 1.2) [11].

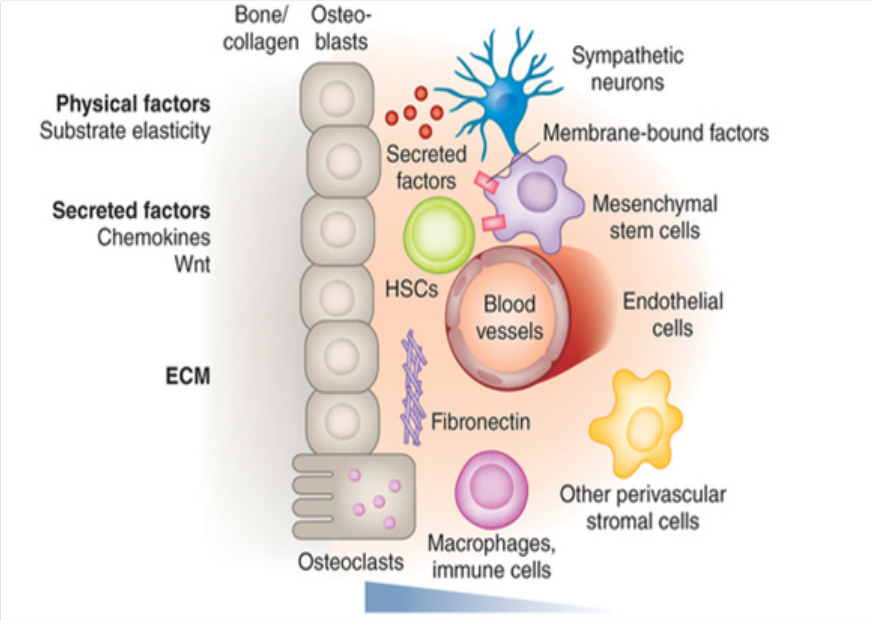

Figure 1.2: Cellular crosstalk and bone formation [4].
Representative schema for the illustration of cellular composition during bone formation. Communication between the cells functioning either direct or indirect contact. Direct contact: cell-cell adhesion, receptor interaction and subsequent signal activation. Indirect contact: cell-cell interaction via secreted factors and subsequent signaling pathway activation.

To mimic the natural bone micro-environment, co-culture systems have been introduced, in which MSCs (from different origin) were cultured together with endothelial cells or macrophages. These research efforts have shown that the co-culture of MSCs with endothelial cells promotes mineralization of MSCs *in vitro* and bone formation *in vivo* [12, 13, 14]. Several studies suggest that macrophages also play a crucial role in bone regeneration, because they are involved in the early responses of wound healing, extracellular matrix formation [15, 16, 17], and secrete a plethora of signaling molecules that are linked to multiple processes during osteogenesis [18, 19].

1.4 Importance of Signaling Mechanisms in Osteogenic Differentiation

In the natural bone regeneration processes, signaling molecules, secreted by cells into the extracellular matrix, act in an autocrine, paracrine, or endocrine manner to affect cell proliferation and osteogenic differentiation of MSCs. Signaling pathways are downstream effectors of these external stimuli and responsible for cell-cell interactions and osteogenic differentiation via specific signal transduction [20]. It has been proven that specific extracellular signaling molecules (i.e. bone morphogenetic proteins (BMPs), transforming growth factor-betas (TGFβs), Wnts, fibroblast growth factors (FGFs), platelet-derived growth factors (PDGF), and insulin-like growth factors (IGFs)), and their activated signaling pathways are directly involved in osteogenic differentiation and bone formation [21]. Understanding molecular mechanisms of bone regeneration (i.e. signaling pathways that are involved in bone regeneration) will help to control cellular responses in cell-based bone tissue regenerative strategies and optimize the efficacy thereof.

1.5 Scaffolds for bone tissue regenerative treatment

In addition to cells, the choice of a biomaterial as a scaffolding structure, is critical for the achievement of successful regeneration. To mimic the 3D micro-environment of bone, several scaffold systems (either from natural or synthetic origin) have been introduced in bone regenerative research. Among these, ceramic-based biomaterials, including calcium phosphates (CaPs; e.g. hydroxyapatite and tricalciumphosphate), and polymer-based organic materials, including collagen and gelatin, are being widely used in the clinic [22, 23, 24]. However, the ideal scaffold system should possess several properties, including biocompatibility, osteoinductive & osteoconductive capacities, ability of fluid transport, delivery of bioactive molecules, surface topography (recognized by cells), degradability and ability to induce signal transduction in cells [25]. So far, none of the developed scaffolds can pass all necessary requirements. Some studies indicate that physical properties of scaffolds (e.g. topography, stiffness) can enhance the osteogenic differentiation of MSCs *in vitro* [26], because cell-biomaterial interactions can promote the activation of specific intracellular pathways and appropriate signal transduction. For example, matrix stiffness can activate the intracellular signaling of MSCs, by help of mechano-transducers: focal adhesion kinase (FAK) and Rho kinase (ROCK), which affect the osteogenic phenotype of MSCs [27]. In view of the promises of tissue engineering, future generation biomaterials should provide controllable signals to loaded MSCs for subsequent osteogenic differentiation. Next, the chemical properties of biomaterials have shown to influence MSC proliferation and osteogenic differentiation [28]. For example, CaP-based biomaterials have high potential to affect osteogenic differentiation of MSCs, because CaP-based biomaterials can absorb osteoinductive proteins and release Ca^{2+} irons [29].

1.6 The objective of this thesis

The research described in this thesis aimed to study cellular and molecular aspects of cell-cell and cell-biomaterial interactions, with emphasis on MSCs and their interaction with both biomaterials and other cell types. More specifically, the following research questions were addressed:

1. Which signaling pathways are involved in osteogenesis and how can the knowledge on these signaling pathways be applied to improve the success of bone regenerative medicine?
2. To what extent do cell culture supplements (with their signaling molecules) affect osteogenic differentiation of human BM-MSCs and AT-MSCs?
3. How does co-culture of human AT-MSCs with endothelial or hematopoietic cells affect the osteogenic signalling and differentiation of MSCs?
4. To what extent does the amount of CaP nanoparticles in a composite collagen/CaP gel system affect MSCs behavior and osteogenic differentiation?
5. Do surface features of CaP-based ceramics affect cellular organization and osteogenic differentiation of AT-MSCs?

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CHAPTER 2

Signaling pathways involved in osteogenesis & their application for bone regenerative medicine

2.1 Introduction

One of the challenges for bone regeneration research is to identify proper strategies for defect repair and regeneration. For orthopedic surgery, the most important problem is how to repair large (segmental) skeletal bone defects originating from various pathological problems, including postmenopausal osteoporosis, resection of metastases due to breast and prostate cancer, and several metabolic diseases [1, 2]. Only in Europe, annually about one million patients need a bone reconstructive surgery and this number is increasing every year [3]. In the US, over sixty million people are expected to be diagnosed with osteoporosis or low bone mass by the year 2020 [4, 5].

So far, bone autografts (i.e. patient's own bone) remain the gold standard for bone regenerative treatments because of the efficacy, safety and immunocom-

patibility of autografts. However, the harvest of autologous bone is associated with several disadvantages, including fracture potential, second surgery at the 'donor' site, size and shape differences, and limitations in volume (max. about 20 cm^3) and quality [2, 6]. An alternative option for bone regenerative treatments is to use bone allograft (donor bone from another human being), but this may lead to transfer of diseases and immunological rejection [7]. Because of the increasing number of bone regenerative treatments and hence demand for reliable grafting material, it is imperative that the multidisciplinary field of biomaterials, in collaboration with the medical device industry, develops bone substitutes (i.e. alloplasts) that meet the clinical and commercial requirements and fully exploit the potential of these bone substitutes to harness the body's own healing capacity for defect healing [5].

2.2 Bone & bone regenerative treatment

Bone is a mineralized tissue rich with inorganic calcium phosphates (i.e. biological apatite), in which the mineralized extracellular matrix provides a natural scaffold for native cells and homeostasis. Bone regeneration involves a series of biological processes, in which multiple cell types (e.g. osteoblasts, osteocytes, osteoclasts, and osteoprogenitors) and multiple extracellular and intracellular signaling networks are involved. Finally, bone tissue possesses an intrinsic regeneration capacity during skeletal development and during bone remodeling throughout the entire life to respond to injury and trauma [3, 8, 9].

Current strategies in bone regenerative medicine are focused particularly on the biomaterial characteristics for the generation of material- and cell-based bone substitutes (alloplasts) that trigger the endogenous healing capacity of the human body [2, 5]. A critical aspect of bone regenerative medicine is to design three-dimensional (3D) scaffolds, in which different cell types (os-

teoblasts, endothelial cells, etc.) can grow and generate the complex structure of bone tissue [5]. Furthermore, new generation of 3D scaffold systems should meet several criteria, such as osteogenic activity, fast integration with surrounding tissue, and biocompatibility in order to provide successful tissue regeneration. To develop an advanced bone substitute, it is necessary to understand the osteogenesis and bone regeneration/remodeling processes in nature. Full understanding of signaling mechanisms responsible for osteogenesis will help to optimize current strategies and create new approaches in bone regenerative medicine. In view of this, it is of utmost importance for the field of regenerative medicine to understand the function of bioactive molecules (e.g. growth factors, cytokines and hormones) and their interplay with endogenous signals, which are driving the cells into osteogenic differentiation. Additionally, the question whether biomaterial properties contribute to cell signaling toward osteogenesis requires attention. Various proteins/growth factors are already being explored in bone regeneration research because of their high therapeutic potential [10]. Although many studies show that such molecules can have a direct and crucial role, their exact molecular mechanisms have not been fully unraveled [11], or these mechanisms are not very well known in the regenerative medicine community. Consequently, the aim of this review is to provide an overview of the molecular mechanisms and complexity of actions of osteogenic molecules and their signaling cascades, which are important for bone regeneration and tissue engineering strategies. The understanding of cross-activation and complex signaling of these molecules will lead in the near future to the design of advanced bone substitute materials. Furthermore, detailed knowledge about regulation of signaling mechanisms in different cell types and molecular consequences of cell-biomaterial interactions will help to control the regeneration of bone defects inside the body.

2.3 Mesenchymal stromal cells for bone regenerative treatment

An adequate supply of bone precursor cells in designed scaffolds is important for efficient bone regeneration. Current approaches using mesenchymal stromal cells (MSCs) in bone regenerative research have received considerable attention, because of the potential of MSCs to differentiate into adipocytes, chondrocytes, osteocytes and several other cell types [12]. It is also known that many tissues, including bone marrow, adipose tissue, periodontal ligaments, dental pulp, muscle tissue, blood vessels and others, contain tissue-specific MSCs [13]. Therefore, the differentiation potential and quality of MSCs from all of these sources needs to be examined before considering these cells as effective cell types for tissue regeneration purposes [10]. In general, the characterization of MSC is based on the expression of surface antigens, such as CD44, CD73 CD90 (Thy1), CD105, CD106, CD166 [14, 15]. Based on this surface antigen specificity, the International Society for Cellular Therapy (ISCT) has defined characterization criteria for MSCs, which include that MSCs require to be positive at least for CD73, CD90, and CD105 and negative to CD45, CD34, CD14, ($< 2\%$) [16]. In addition, MSCs need to have several other characteristics, such as adherence to plastic and multilineage differentiation capacity [14, 15, 17].

Because of their high osteogenic potential, MSCs isolated from bone marrow (BM-MSCs) are the most popular cell population in bone tissue regeneration research [18]. Despite the fact that BM-MSCs meet all of the criteria, as indicated above, several disadvantages related to the isolation of BM-MSCs (especially the invasive harvesting methods) have stimulated scientists to search for alternative sources of MSCs. Recently, research efforts have focused on MSCs isolated from adipose tissue (AT-MSCs) because of their multi-lineage differentiation capacity and large similarity to BM-MSCs in terms of surface antigen expression, osteogenic differentiation, and adherence to the plastic

[16]. However, in contrast to BM-MSCs, AT-MSCs are much easier to obtain, donor morbidity is very low and large numbers of cells can be harvested from relatively small volumes of adipose tissue. AT-MSCs are also more stable after expansion and after cryopreservation compared to BM-MSCs [12]. Further, it has been proven that AT-MSCs are able to secrete many growth factors, most of which have angiogenic as well as osteogenic capacities [14, 19]. For instance, it is known that AT-MSCs produce fibroblast growth factor-2 (FGF-2) and vascular endothelial growth factor (VEGF), which are crucial for angiogenic stimulation, but the same growth factors have also osteogenic activity [20]. Finally, AT-MSCs have shown several other features such as self-renewal ability, high proliferation capacity and potential of osteogenic differentiation [15]. In view of this, it becomes significant to study first the key signaling pathways, which lead osteoprogenitor cells to differentiate into osteoblasts, in order to understand the potential of other MSCs (from different origin) to pass complete osteogenic differentiation [21].

2.4 Growth factors, hormones & their signaling activity

Growth factors (e.g. cytokines, hormones) are proteins, which regulate a large variety of cellular processes. They act as signaling molecules between cell-cell interactions and usually interact with their specific receptors on the surface of the target cell. Growth factors are secreted by cells into the extracellular matrix and act in an autocrine, paracrine, or endocrine manner to affect cell proliferation, differentiation and maturation. Thus, growth factors regulate cell signaling from an extracellular level until target gene expression and protein synthesis in cells [5]. Every signaling molecule is responsible for a particular signaling pathway activation and signal transduction. There are two general signaling mechanisms that can transfer external signals to the

nucleus. The first mechanism is typical for exogenous lipophilic signaling molecules, which are able to penetrate cellular membrane and interact directly with transcriptional regulators. The second mechanism is typical for hydrophilic molecules (i.e. growth factors and cytokines), which are not able to penetrate the cell membrane. However, these molecules are intercepted by trans-membrane receptors, and transmit a second message or secondary reaction into the cytoplasm. At the intracellular level, this second message stimulates transcription factors (second messenger molecules) to be activated via mechanism of posttranslational modification, e.g. phosphorylation [22]. Within the nucleus, the active (phosphorylated) factors (ee.g. transcription factors, co-activators, co-repressors) interact with regulatory DNA motives (i.e. with promoter, enhancer or silencer regions) giving rise to transcription (more detailed description of signal transduction mechanism see reference [22]). The mechanism of many growth factors/cytokines from extracellular level until target gene transcription has been already unraveled and it has been proven that many of them (i.e. bone morphogenetic proteins (BMPs), transforming growth factor-betas (TGFβs), Wnts, fibroblast growth factors (FGFs), platelet-derived growth factors (PDGF), and insulin-like growth factors (IGFs)), are directly involved in osteoblast activity and local bone formation [23].

2.5 Molecular pathways & osteogenesis

2.5.1 BMP/TGF-β pathway

BMP/TGF-β pathways are major signaling cascades responsible for osteogenesis [24, 25]. BMP/TGF-β signaling is involved in the vast majority of cellular processes that are responsible for bone formation during mammalian development [26, 27]. The disruption of BMP/TGF-β signaling causes multiple bone disorders such as tumor metastasis, brachydactyl type A2, and

osteoarthritis (Table 2.1, Table 2.2) [28].
BMPs and TGF-βs are cytokines that belong to the TGF-β superfamily, which includes approximately 40 members divided in several subgroups: BMPs, TGF-βs, Nodal and Activins [29, 30]. In order to start signaling, BMP/TGF-βs interact with BMP or TGF-β specific type 1 and type 2 serine/threonine kinase receptors [31, 33]. Accordingly, the BMP/TGF-β regulated cascade is transmitting the signal into the cytoplasm via both canonical (i.e. Smad-dependent pathways; BMP/TGF-β ligands, receptors and Smads) and non-canonical pathways (i.e. Smad-independent signaling pathways; e.g. p38, mitogen-activated protein kinase: MAPK (Figure 2.1) [24].

In canonical pathways, 8 different types of Smad proteins are involved. According to their function Smads are divided in three classes. The first class is the receptor-regulated Smad family or R-Smad (Smad 1, 2, 3, 5 and 8; Figure 2.1). Smad 1, and 5 are usually activated by BMP extracellular signals, and Smad 2 and 3 are activated by TGF-β extracellular signals [37]. The second class is common-mediator Smad (co-Smad), which includes only Smad 4. Smad 4 forms a complex with co-Smads, penetrates into the nucleus, then interacts with DNA promoter region and as a transcriptional co-activator participates in transcription. The third functional class of Smads is the inhibitory Smad family or I-Smads (Smad 6 and 7), which are negative regulators for BMP/TGF-β-Smad signaling cascade. I-Smads also mediate receptor inactivation of other Smad classes (class 1, 2) [38]. Non-canonical TGF-β signaling also participates in osteoblast differentiation, osteoprogenitor cell proliferation and bone formation [41]. This pathway includes many signaling molecules, which mainly belong to mitogen-activated protein kinase family, MAPKs (for a more detailed description of MAPK cascades see references [38, 41]). The MAPK cascade is a widely studied signaling pathway, which has been shown to be involved in important cellular programs, such as cell differentiation, movement, division and death [39]. In the context of bone regeneration, the P38/MAPK activation cascade has a crucial function for an efficient induction of osteoblast differentiation. This cascade (P38/MAPK)

is involved in alkaline phosphatase (ALP) and osteocalcin (OC) expression in osteoblastic cells, providing evidence that these MAPKs have distinct roles in regulating osteoblast differentiation (Figure 2.1 , Figure 2.2) [42].

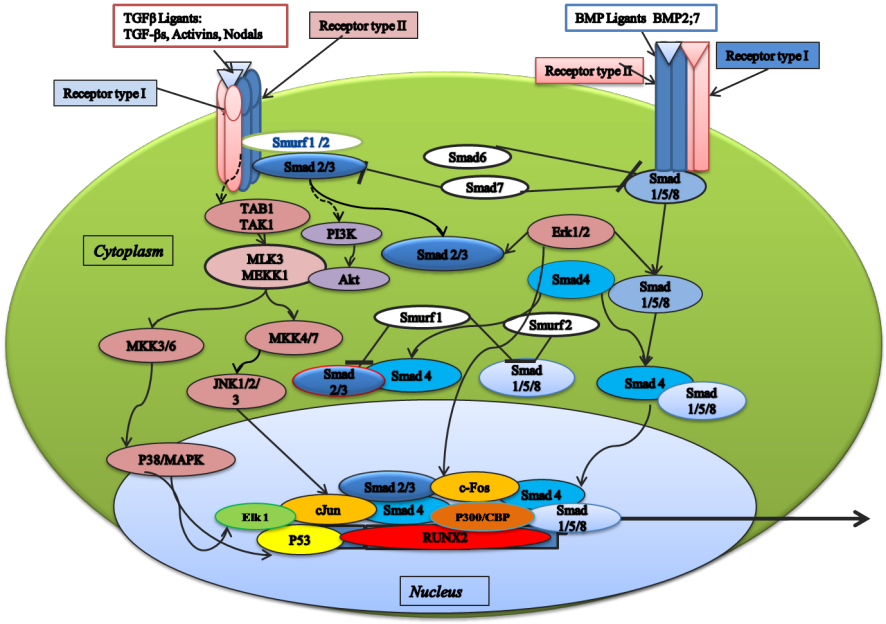

Figure 2.1: Canonical/non Canonical TGFβ/BMP2 Pathways

TGFβ regulate heteromeric complex formation between TGFβ specific type I and type II serine/threonine kinase receptors [31, 32, 33]. BMPs start signaling via the activation of two types of serine/threonine kinase receptors (BMP type I and BMP type II) [34, 35, 36]. This complex formation is important and sufficient for Smad and other non-Smad signaling activation, because this complex leads to the phosphorylation and activation of the type I receptors in cytoplasmatic site [31]. The signal transduction regulated by BMP/TGFβ is directed to both canonical (Smad-dependent pathways; BMP/TGFβ ligands, receptors and Smads) and non-canonical signaling pathways (Smad-independent signaling pathway; e.g. p38, mitogen-activated protein kinase (MAPK) pathway) to the cytoplasm [24]. The canonical BMP/TGFβ pathway starts signaling in the cell surface via the interaction with specific receptors. In the cytoplasm activated receptors are interacting with R-Smads (Smad 1, 5 and 8). With the same mechanism the TGFβ extracellular signals can be

activated with Smad 2 and 3 [37]. Prior to phosphorylation, R-Smads (Smad 1, 2, 3, 5, 8) form heteromeric complexes with Smad 4 (common mediator Smad class or co-Smads). After complex formation the entire complex is translocated into the nucleus and accumulated there prior to regulate the transcription of target genes in combination with other transcription factors [37, 38]. The Smad 6 and 7 (inhibitory Smads or I-Smads) are negative regulators of BMP/TGFβ - Smad signaling and mediate receptor inactivation of other two other Smads classes [37, 38]. In non canonical signaling pathways, the TGFβ activation kinase 1 (TAK1) and TAK1 binding protein 1 (TAB1) play a crucial role for upstream signal activation followed by MKK3/6 and P38/MAPK. TAK1 regulates MKK3 and p38/MAPK protein levels in the entire MAPK cascades [24, 39]. Therefore, the TGFβ induction in either Smad dependent or independent pathways translocates the signal into the nucleus, leading to Runx2 transcriptional gene control [40].

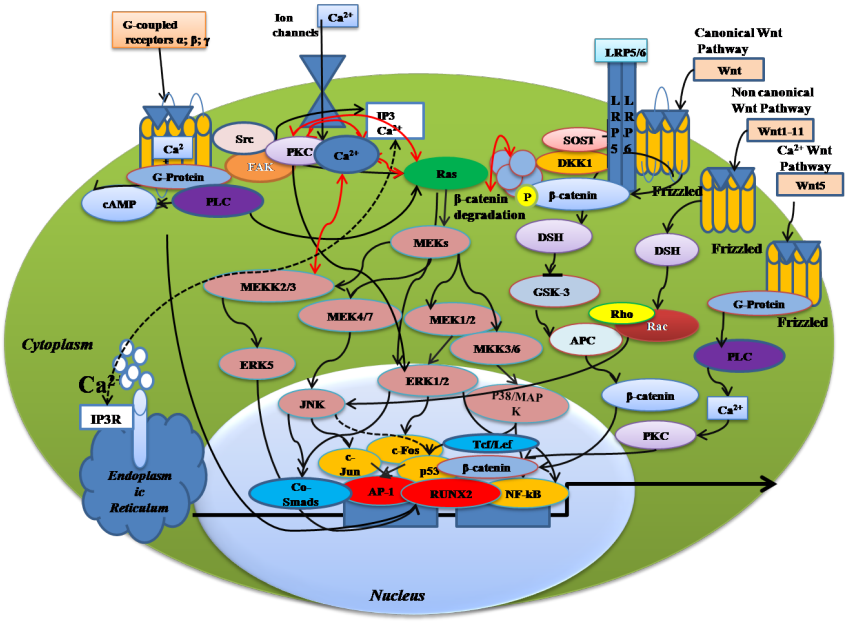

Figure 2.2: Ca^{2a+} signaling and Wnt signaling.

Ca^{2a+} signaling: Runx2 mediates ATP-dependent Ca^{2a+} influx via the calcium channels with the help of protein kinase C (PKC), Src family kinase, protein kinase A (PKA), and further via the phosphorylation of focal adhesion kinase (FAK) and proline-rich tyrosine

kinase 2 (PYK2) [43]. The cytoplasmic Ca^{2a+} promotes phospholipase C (PLC) activity and inositol-1,4,5-trisphosphate (IP3) signaling [44]. The increase of cytoplasmatic Ca^{2a+} promotes subsequent PKC and Ras activity, which are involved in activation of the MAPK pathway via the ERK1/2, JNK activation and upregulation of c-fos and c-Jun expression [45]. The c-fos gene product together with c-Jun protein forms the activator protein-1 (AP-1). AP-1 is a transcription factor that binds to the promoter region of mechano-sensitive genes [44]. This mechanical stimulation leads to upregulation of several growth factors, such as insulin-like growth factor (IGF I and II), vascular endothelial growth factor (VEGF), TGFβ, and BMP2 and BMP4, which start signaling on the cell surface [44, 46, 47]. On the cell surface, Ca^{2a+} interacts with G-protein coupled receptors (GPCRs) and activates this machinery. The most widely described GPCR pathway involving Ca^{2a+} is the calcium sensing receptor machinery (CaSR). Also PLC can be activated by GPCRs, resulting in synthesis of IP3 and its signaling activation [48]. The PKA signaling activated by Ca^{2a+} can induce FGF-2 gene expression (identification in cementoblasts) [49]. In osteoblasts, Ca^{2a+} induces phosphorylation of ERK1/2. This phosphorylation is significantly reduced in the presence of a CaSR antagonist [50].

Wnt molecules activate at least three distinct intracellular signaling cascades: Wnt/β-catenin pathway (canonical), Wnt/Ca^{2a+} pathway (non canonical), and Wnt/planar polarity pathway. The ligands (Wnts) of canonical pathway first form a complex with FZD and low density lipoprotein (LDL) related protein 5 (LRP5) or LRP6 receptors that lead to signal initiation, and translocate the signal into the cytoplasm. Within the cytoplasm, this complex inhibits the activity of glycogen synthase kinase 3 (GSK-3) with the help of several proteins (i.e. Disheveled, Axin, and Frat-1). Wnt pathway-dependent/GSK3 inhibition helps to stabilize β-catenin level in the cytoplasm [51]. GSK-3 inactivity blocks the phosphorylation of β-catenin, which prevents degradation of β-catenin in the cytoplasm leading to its translocation into the nucleus. In the nucleus, β-catenin first forms a complex with Tcf/Lef transcription factors (T-cell/lymphoid enhancer transcription factor family) and activates target gene transcription [25, 51]. The Wnt/planar polarity pathway activates Rho/Rac GTPases and Jun N-terminal kinase (JNK) to modulate cytoskeletal organization and gene expression [52].

Table 2.1: Most Important BMP Family Members.

Name	Synonyms	Main Function
BMP1	None	Metaloprotease activity Acts on pro-collagen type I, II, III; Cartilage development ; BMP1 does not belong to the TGFβ family of proteins
BMP2	BMP 2A	Induces bone and cartilage formation, osteoblast differentiation. Plays a role in cardiac morphogenesis
BMP3A	Osteogenin	Induces bone and cartilage formation.
BMP3B	GDF-10	Unknown biological function, it may play a role in osteoblast differentiation, augmenting BMP 2 activity.
BMP4	BMP2B; BMP2-B1, BMP2-B2, BMP2-RS1	Induces bone and cartilage formation, involved in regulation of teeth formation, limbs and bone formation in mesoderm, fracture repair.
BMP5	None	Induces bone and cartilage formation.
BMP6	Vgr-1, Vgr-1 related protein	Induces bone and cartilage formation and involved in iron homeostasis control.
BMP7	OP-1(osteogenic protein 1)	Induces bone and cartilage formation, involved in calcium regulation and bone homeostasis, key role in osteoblast differentiation. Induces the production of Smads. May act as an osteo-inductive factor responsible for epithelial osteogenesis. Key role in renal development and repair.
BMP8A	OP-2(osteogenic protein 2)	Induces bone and cartilage formation, involved in calcium regulation and bone homeostasis. May act as an osteo-inductive factor responsible for epithelial osteogenesis.
BMP8B	OP-2(osteogenic protein 2)	Stimulates cartilage and bone formation, implicates in calcium regulation and bone homeostasis.
BMP9	GDF-2	May be involved in bone formation, involved in chondiriogenesis
BMP10	None	Plays a crucial role in trabeculation of embryonic heart
BMP11	GDF-11	Involved in patterning of both mesodermal and neural tissues and establishing the skeletal muscle. Act globally to specific positional identity along the anterior/posterior axis.
BMP12	GDF- 7	Induces the formation of tendon and ligament tissues.
BMP13	GDF-6	Plays a role in cartilage homeostasis, involved in the embryonic skeletal development, and formation if tendon-like tissue.
BMP14	GDF-5	Essential for limb cartilage and limb-joint formation. Involved in embryonic skeletal development.
BMP15	None	Role in oocyte and follicular development.

Table 2.2: Most Important TGFβ BMP Family Members.

Name	Synonyms	Main Function
TGFβ1	None	Cell growth, proliferation, differentiation apoptosis
TGFβ2	None	Regulates cell proliferation, growth, differentiation, and motility. Involved in adipogenesis, chondrogenesis, embryogenesis, tissue remodeling, wound healing and tumor formation.
TGFβ3	None	Regulates cell proliferation, growth, differentiation, and motility. Involved in adipogenesis, chondrogenesis, embryogenesis, tissue remodeling, wound healing and tumor formation Cellular adhesion, extracellular matrix formation.
TGFβ4	None	Essential for left-right asymmetry determination of organ systems.

2.5.2 Ca^{2a+} signaling

Ca^{2+} signaling plays an important role in the osteoblast differentiation process. Via diverse signaling pathways, Runx2 plays the role as a mediator for ATP-dependent Ca^{2+} influx through calcium channels. The increasing level of cytoplasmic Ca^{2+} promotes phospholipase C (PLC) activity and inositol-1,4,5-trisphosphate (IP3) signaling. The increasing level of cytoplasmic Ca^{2+} promotes phospholipase C (PLC) activity and inositol-1,4,5-trisphosphate (IP3) signaling (39). This signaling leads to a release of Ca^{2+} from intracellular stores (mainly from the endoplasmatic reticulum) [53]. The increased cytoplasmatic Ca^{2+} further promotes upregulation of the transcription factor AP-1, which binds to the promoter region of mechano-sensitive genes. This leads to upregulation of several growth factors, such as insulin-like growth factor (IGF 1 and 2), vascular endothelial growth factor (VEGF), TGFβ, BMP2, and BMP4 (for more details on mechanism of Ca^{2+} signaling see reference [44]). These growth factors (i.e. VEGF, TGFβ and BMPs) start signaling on the cell surface via autocrine and paracrine mechanisms (i.e. cell-cell communication and signaling) [46, 47]. Full screening of the mechanism

of Ca^{2+} signaling will help to improve the understanding of the biological process of osteogenesis and bone regeneration. The mechanism of Ca^{2+} signaling may also explain how CaP particles can enhance osteoblastic activation and high mineralization capacity of osteoprogenitor cells [54]. Moreover, it is proven that CaP crystals fail to induce expression of an osteoblast characteristic set of genes when upstream activators of ERKs (MAPK signaling) are blocked (identification in mouse embryonic fibroblasts), which provides an evidence about the relation between Ca^{2+} and MAPK signaling (Figure 2.2) [55].

It is also known that Ca^{2+} channels can be activated by CaP crystals (identification in a mouse embryonic fibroblast cell line) and by free Ca^{2+}, dissolved from hydroxyapatite, which leads to an increase of osteopontin (OP), bone sialoprotein (BSP) and ALP expression [56]. It is proven that Ca^{2+} is a ligand for G-protein coupled receptors (GPCRs) [48]. These GPCRs have been identified in both osteoblasts and MSCs [57]. Several other receptors, which are responding to Ca^{2+} fluctuations, belong to this GPCR family. Members of this family are metabotropic glutamate receptors and their activity has been correlated with osteoblast function [58]. Ca^{2+} can also enter to the cytoplasm via the gap junction, hemi-channels or via the activation of Notch signaling pathway [59].

2.5.3 Wnt pathway

Wnts (for wingless) are a large family of ligands for the membrane-spanning frizzled (FZD) receptors. Frizzled receptors belong to G protein coupled receptor family that are activating only with Wnt ligands. Wnt pathways are involved in various cell activities, such as proliferation, growth, differentiation, fate determination, polarity, migration, and cell death. Wnt signaling plays an important role in osteoblast differentiation and mineralization processes. Wnts activate at least three distinct intracellular signaling cascades: Wnt/β-catenin pathway (canonical), Wnt/Ca^{2+} pathway (non-canonical),

and Wnt/planar polarity pathway (Figure 2.2 and 2.3) [60, 61]. There are several studies, which indicate that canonical Wnt pathways play a critical role in bone formation, because of their involvement in the expression of osteoblast-specific gene markers [25]. The Wnt/planar polarity pathway modulates cytoskeletal organization via the activation of osteoblast-specific gene expression [51, 52].

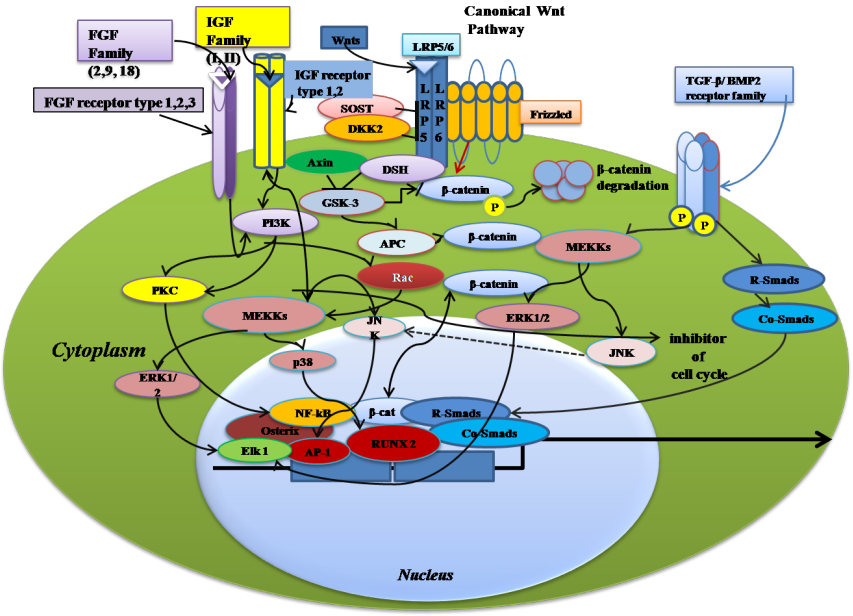

Figure 2.3: Signaling networks involved in osteoblast differentiation and molecular crosstalk between signaling pathways.

FGF signaling: The members of fibroblast growth factors (FGFs) family can bind to seven different isoforms of FGF tyrosine kinase receptors (FGFRs).

IGF signaling: It is known that IGFs can activate the signaling via the activation of ERK or phosphatidylinositol 3-kinase (PI3K) pathways. IGFs involved also in osterix upregulation via the BMP independent pathway, which is followed by PKC activated pathway and RUNX2 indirect activation [25].

2.5.4 FGF pathway

The FGF pathway has been shown to be involved in osteogenesis as well as several other cellular processes, such as angiogenesis and wound healing [62]. In osteoprogenitor cells, the role of the FGF pathway is critical in the controlling process of endochondral and intramembranous signaling [63]. Normally, the FGF pathway stimulates osteoblast differentiation and inhibits osteocyte differentiation. Therefore, in osteoblast maturation process, FGF signaling has stage-specific effects [64]. To date, a total of 23 FGF family members are known that are able to interact with 4 different tyrosine kinase receptors. It has been shown that Runx2 is phosphorylated and activated by FGF2 via the MAPK pathway, which suggests an important role for FGF2 in the regulation of Runx2 function and bone formation (Figure 2.3) [65]. Further, it is known that FGF2 is secreted by AT-MSCs [25]. The expression of FGF9 and FGF18 plays a predominant role during embryonic skeletogenesis [51]. Several studies indicate that FGFR2 (fibroblast growth factor receptor 2) positively regulates bone growth and the anabolic function of osteoblasts [66, 67]. The FGF pathway activation via FGFR3 regulates cell growth and differentiation of proliferating chondrocytes, whereas in differentiated osteoblasts it regulates bone density and cortical thickness [25].

2.5.5 Insulin-like growth factor (IGF) & signaling

IGF signaling is one of the important signaling pathways involved in proliferation and differentiation of osteoblasts (Figure 2.3). The IGF family consists of two members, namely IGF1 and IGF2. IGF2 is the most abundant growth factor in the bone [68]. Both IGFs are expressed in osteoblasts and have similar biological characters. They are responsible for stimulation of osteoblast differentiation and bone matrix deposition. Their function is crucial in expression of collagens and non-collagenous proteins [25]. However, in contrast

to IGF2, IGF1 does not influence the proliferation and differentiation of MSCs toward osteoblasts, but it is an important molecule for longitudinal bone growth and the maintenance of bone mass [69].

2.5.6 Platelet derived growth factor (PDGF) & signaling

PDGF is an extracellular signaling factor, from which five biologically active isoforms are known. One of the members of this PDGF family is the VEGFA subfamily [70]. Several studies indicate that PDGF signaling plays an important role in the control of various cell functions in skeleton [71, 72]. However, the network of PDGF signaling in MSCs has not been fully investigated yet.The depletion of the β-PDGFR gene in MSCs increases ALP activity and the expression of osteocalcin, BMP2, Runx2 and osterix at the mRNA level [73]. More recent studies indicate that the pharmacological inhibition of PDGFR decreases MSC proliferation, but does not affect osteoblastic differentiation [74].

2.5.7 Notch pathway, cellular crosstalk & signaling

Notch pathway is traditionally known for its ability to facilitate short-range signaling between neighboring cells (cellular communication), coordinating spatial and temporal regulation of cell fate during embryonic development of tissues and organisms. Notch pathway is involved in osteoblast differentiation via canonical (BMP/Smads) as well as non-canonical (TGFβ/MAPKs) pathways [75]. Notch signaling regulates distinct cellular programs in different cell types. Notch signaling directly inhibits osteoblast differentiation and indirectly influences osteoclast differentiation [76]. This pathway is an important sign for osteoclast maturation and function. There is experimental evidence, which shows that Notch signaling prevents the differentiation of os-

teoclast precursors into mature osteoclasts [76, 75]. For the Notch signaling cascade, cell-cell interactions are required. At the nuclear level, Notch signaling is involved in up-regulation of bone related genes. Notch signaling can also induce severe osteosclerosis, because it can inhibit Runx2 transactivation [25].

2.6 Transcription factors

Runx2 (also called Core-binding factor alpha, Cbfa1) is an essential transcription factor for osteoblast differentiation, matrix production and mineralization during bone formation [77, 78], because Runx2 regulates downstream genes that determine the osteoblast phenotype and function during skeletogenesis [79]. Runx2 is the key transcription factor in the nuclear microenvironment that controls the expression of osteogenic genes in response to physiological signals [5, 25]. In particular, the Runx2 is the master regulator for expression levels of osteogenic marker genes such as alkaline phosphatase (ALP), osteopontin (OPN), type I collagen, bone sialoprotein (BSP), and osteocalcin (OC) [79, 81]. Runx2 regulates the expression of Osterix (OX), which is a key transcription factor for osteoblast differentiation [43]. In the nucleus, Runx2 binds to osteoblast-specific cis-acting element (OSE2), which is present in the promoter region of several osteoblast-specific genes [69, 79, 82]. Runx2 regulates BMP signaling from the cell surface until the target gene expression (BSP, ALP, OC, etc.) in the nucleus [78, 83]. Therefore, an appropriate mRNA dosage of Runx2 is crucial for normal bone development [84]. However, in mature osteoblasts, Runx2 needs to be suppressed in order to form mature bone [78].

Runx2 expression is controlled by various extracellular signals including BMP and fibroblast growth factor (FGFs) pathways [78]. Transcriptionally, Runx2 is upregulated by BMP2 via the transduction of Smad 1, 3, 5 [5].

Overexpression of Runx2 may cause several disorders. It has been shown that overexpression of Runx2 can inhibit adipogenesis and reduce lipid droplet formation in AT-MSCs. This phenomena result in early osteoblast differentiation and osteoblastic gene expression (ALP and mineral deposition) activity in AT-MSCs [84]. A forced expression of Runx2 in non-osteoblastic cells can also promote expression of the major osteoblast specific genes [79]. BMP/TGF-β pathway is also involved in Runx2 stabilization via the activation of Smad 5. The Erk activated pathway promotes Smad-induced Runx2 acetylation and stabilization. All these results indicate that Erk signaling (from BMP/TGF-β) increases Runx2 stability and transcriptional activity [78].

Osterix (OX) is a transcriptional regulator for the final stages of bone formation. Osterix is a zinc finger protein, which contains specific domains responsible for osteocalcin and collagen type 1 gene activation. OX-null osteoblast precursors can express only chondrocyte markers, such as Sox9 and Col2a1, which means that OX-null osteoblast precursors can neither be differentiated into osteoblasts nor deposit bone matrix. Thus, without OX bone formation cannot occur [69]. These data prove that OX is a downstream regulator for Runx2 able to induce osteoblastic differentiation in osteo-progenitor cells [43]. However, OX can be induced by other signaling pathways, which are acting in parallel and independent of Runx2. It has been shown that Runx2 is not sufficient for the BMP-2 mediated OX induction, because MAPK signaling pathways serve as points where the BMP-2 can effect directly on OX expression [25].

2.7 The application & future perspective of signaling pathways in bone regenerative medicine

The induction of osteogenic differentiation is the basis of bone regeneration, which is only possible via the activation of responsible signaling mechanisms. To be able to develop advanced bone substitute materials, it becomes imperative to implement the initiation of those signaling mechanisms in the strategies of bone regenerative research and therapy. Therefore, in bone regenerative medicine, the involvement of knowledge about cell signaling becomes an important concept.

The natural matrix of bone, which is rich with several signaling molecules secreted by different cell types (Figure 4a), is able to promote osteogenesis [85]. These molecules activate entire signaling cascades inside cells as described in the previous sections. An effective implementation of cell signaling knowledge for the improvement of bone regenerative treatments might rely on one or more of the following possible strategies:

- incorporating signaling molecules via loading or genetic modification of stromal cells
- exploiting cell-cell interactions
- optimization of material surface properties
- utilization of material degradation products

2.7.1 Incorporation of signaling molecules or genetic modification of stromal cells

From the BMP family members, the most appealing osteoinductivity was observed for BMP2 and BMP7. These particular growth factors already have become a modulating tool for bone regenerative research, aimed to develop therapeutic concepts for the restoration and treatment of skeletal defects. Furthermore, BMP2 and BMP7 are commercially available for clinical use as human recombinant proteins, manufactured via mammalian cell expression systems [85]. However, the disadvantage of using BMPs in bone regeneration is the required high dosage and the related clinical side-effects [86]. The perspective of bone regeneration via the help of signaling molecules should be directed to the improvement of expression quality of these proteins inside the body, preferably via material-based release systems with optimized control on the release of loaded BMPs.

Alternatively, with the help of advanced gene transfer technologies in the near future, it will become possible to control MSCs to express an appropriate dosage of signaling molecules during the regeneration process [85, 87]. Despite the fact that several studies already show the benefit of gene transfer technologies as a powerful strategy in bone regeneration therapy, the step from experimental research to clinical practice still requires hurdles from a scientific, industrial, and ethical perspective.

Genetic engineering technologies have another potential to be involved in bone regenerative research and therapy. For example, it will be beneficial to identify and knock out genes, which are negatively regulating osteogenesis. Recently, because of direct effect on MSCs differentiation into osteoblasts, Wnt signaling has attracted interest for applications in bone regenerative research. Therefore, the identification of target molecules from Wnt pathway could help to promote MSCs into osteoblast differentiation. Several studies show that pharmacological inhibition of Src (Sarcoma rous cellular

oncogene homolog) signaling (Src is a molecule from Wnt signaling networks) increases Wnt canonical signal and early osteoblast differentiation in MSCs. This observation makes Src a potential target for the control of MSC differentiation processes [88]. Osteocytes can control bone mass via the production of negative regulators of Wnt/β-catenin pathway, such as Dkk1 and Sclerostin (SOST). SOST is an inhibitor of osteoblast activity. Based on this observation, it would be possible to control osteoblast activity during bone regeneration using Dkk1 and SOST as target molecules. Several preclinical trials have focused on targeting Dkk1 and SOST for the treatment of postmenopausal osteoporosis and for clinical trials an antibody against to SOST is already commercially available [89].
Still, the field of gene transfer technologies and gene therapy requires more research to avoid possible complications, including previously observed patient death [90].

2.7.2 Cell-cell interactions

Angiogenesis and vascularization are crucial for bone regeneration and fracture healing, because the blood supply delivers oxygen and nutrients into a defect site. Vascularization is one of the main issues in the fields of bone regenerative research [91]. In bone regeneration processes, endothelial cells plays an important role, because they (endothelial cells) also produce osteoinductive molecules, such as BMPs, which are directly involved in MSCs osteogenic differentiation [92, 93]. A recent in vivo study (in a BMP2 reporter transgenic mice model) showed that the high expression level of BMPs was generated by endothelial cells [94]. This observation and numerous other *in vitro* co-culture studies proved that osteogenic differentiation of MSCs significantly increases in the presence of endothelial cells, which is a result of cellular crosstalk and substantial signaling activation [95, 96]. The perspective of these observations will lead to an advanced design of co-culture (cell combination) systems with improved osteogenic as well as angiogenic

capacities [91, 97]. However, the concept of cell-cell interactions is not only based on endothelial cell and MSC combinations, because in the natural bone micro-environment many other cell types are present (Figure 2.4a) [98].

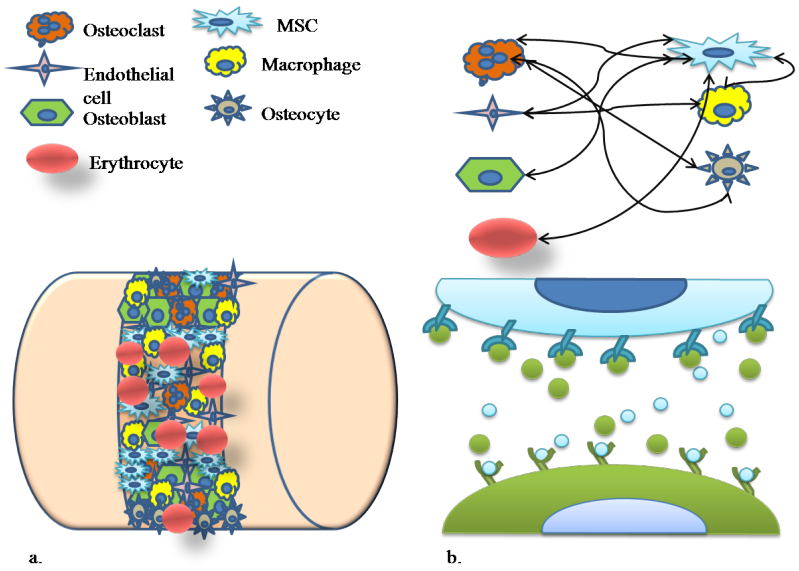

Figure 2.4: a. Cellular composition in defect site of bone b. Cell-cell interaction and intracellular signaling.

(a) The cellular composition in the defect site of bone during wound healing process. The cellular micro-environment allows cells to communicate via expressed bioactive molecules (cellular crosstalk). (b) These bioactive molecules can be involved in activation of signaling networks of other cell types (such as crosstalk between endothelial and bone progenitors) [93].

Recently, it has been found that monocytic exosomes, co-cultured with MSCs, can stimulate upregulation of osteogenesis-related genes (Runx2, BMP2, osteocalcin) via cellular crosstalk [99].This particular study did not describe the pathways that are responsible for molecular crosstalk and osteogenic signaling. The future perspective of this field (i.e. co-culture between different cell types) will be the improvement of detailed molecular screening of cel-

lular crosstalk (Figure 2.4b). Further, thorough testing of such co-culture approaches needs to be carried out and the influence of reliable biocompatible scaffolds needs to be evaluated.

2.7.3 Optimization of material surface properties

Molecular screening of cell behavior/osteoinductivity, in applications with synthetic biomaterials, becomes also very important in the field of bone regenerative research. To mimic bone microenvironment, synthetic biomaterials need to meet several criteria, such as porosity (similar to bone), interconnectivity, biocompatibility, mechanical properties and chemical stability [100, 101]. Several studies indicate that synthetic biomaterials can improve the extent of MSC proliferation, differentiation and mineralization [102, 103, 104]. However, only a few studies report about molecular mechanisms of cell-biomaterial interaction and signaling [105, 106, 107]. Nevertheless, the molecular screening of cellular response to material properties will contribute to determine the efficacy of biomaterials for eventual clinical use.

One of the most commonly used biomaterials in bone implantology is titanium, because of its biocompatibility and reliable mechanical properties. Despite the fact that titanium is in clinical use already for quite a long time, the mechanism of cellular osteointegration and molecular response of cells remains largely unclear [108]. Furthermore, several studies indicate that topographical cues on the surface of biomaterials affect cell behavior and osteogenic activities, a topic that has also been explored for titanium surface topography can increase osteoinductivity of contacting cells [109, 111]. More particularly, it was shown that a rough titanium surface enhances the secretion of osteoblastic phenotype related proteins, e.g. ALP, osteocalcin, osteoprotegrin and TGF-β [105, 106]. Finally, it was demonstrated that the roughened titanium surface (sandblasting with grits of 0.25-0.5mm) induces an upregulation of genes responsible for osteogenesis (BMPs and Smad fam-

ily), for transcriptional regulation (NF-kB), for angiogenesis (VEGFs), for extracellular matrix formation (collagen and surface markers) and several other genes including IGF1R, IGF2, FGF2 [108]. This observation and further fundamental investigation of osteogenesis-related gene expression profiles will help to increase the quality of newly designed biomaterials with superior performance in terms of osteoinductivity and osteogenicity.

2.7.4 Material degradation products

Calcium phosphates (CaP) are a very popular family of bioactive materials, which are used in bone tissue regenerative research. As an appropriate scaffold material and cell carrier, CaP biomaterials have been applied in numerous *in vitro* and *in vivo* studies [112]. Several clinical trials showed that CaP scaffolds in combination with pre-cultured MSCs have substantial healing capacity for bone defects [7, 113]. In order to understand the biological interaction and osteoinduction of MSCs in contact with CaP, molecular screening of cell/CaP biomaterial interaction effects has been performed. This screening showed that Ca^{2+} ions released from scaffolds might be responsible for inducing osteogenic differentiation in MSCs, as demonstrated by increased ALP and BMP2 expression [48]. Additionally, β-calcium silicates (β-CS) recently received major attention in the field of bone regenerative research, because it was demonstrated that a composite scaffold, consisting of β-CS and PDLGA (poly-L-lactic-co-glycolic acid), was able to upregulate the osteogenic differentiation of MSCs by help of ionic extracts released from the scaffold. In particular, it was shown that bioactive ions (released from β-CS/PDLGA scaffold) were able to enhance cell viability, ALP activity, and calcium mineral deposition. Finally, at mRNA level, an upregulation of MAPKs, Erkl/2 (from TGF-β pathway), and Runx2 was observed. These data suggest that β-CS/PDLGA composite material can promote bone regeneration [114]. Future characterization and evaluation of these materials in cell-biomaterial concepts, together with detailed knowledge about the molec-

ular response of cells, will bring those materials more closely to preclinical and clinical bone regenerative applications.

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CHAPTER 3

Osteogenic signaling of human BM-MSCs & AT-MSCs cultured in PL- or FBS- supplemented media

3.1 Introduction

Due to the continuously aging population, with an increasing incidence of bone-related problems (i.e. osteoporosis, bone tumors and skeletal injuries), new strategies for the treatment of bone defects become of utmost importance. Over the last decades, bone tissue regenerative approaches increasingly focused on cell-based strategies, which are based on *in vitro* expansion of mesenchymal stromal cells (MSCs), steering them into the osteogenic lineage and their subsequent seeding on a scaffold for *in vivo* application [1, 2].

MSCs isolated from different tissues, including bone marrow (BM) and adipose tissue (AT), have been characterized and pre-clinically applied in several studies [3, 4]. Many studies reported that BM-MSCs are able to differentiate into osteoblasts *in vitro* and *in vivo* [5, 6, 7]. However, the harvest of bone marrow cells is an invasive procedure with high risk of complications.

Moreover, the amount of harvested cells is often relatively limited [8].

Recently, AT-MSCs have become more popular in bone regenerative research, because of their easy accessibility and relatively large yield [4, 9, 10]. In order to apply AT-MSCs in bone regenerative research, a characterization based on proliferation and osteogenic differentiation capacity is recommendable. Several studies have indicated that AT-MSCs proliferate much faster than BM-MSCs, and show substantial osteogenic differentiation capacity *in vitro* [11, 12, 13]. Although the osteogenic potential of AT-MSCs is proven by aforementioned studies, the underlying molecular mechanisms of osteogenic differentiation seem to be different from BM-MSCs [14]. For example, AT-MSCs do not respond well to bone morphogenetic protein 2 (BMP2) [15, 16], while BMP2 is known to be the most powerful growth factor involved in the osteogenic differentiation of BM-MSCs [17]. In addition, it is proven that the expression level of some of the receptors involved in BMP/TGF-β pathways are downregulated or even not expressed in AT-MSCs [18]. This indicates that molecular mechanisms of osteogenic differentiation of AT-MSCs and BM-MSCs are different [9, 19]. In view of this, it seems logical to study the osteogenic differentiation of MSCs (i.e. AT-MSCs and BM-MSCs) at a molecular level, focusing on the osteogenic signaling pathways (e.g. the BMP/TGF-β and Wnt-pathways), and the natural bone regeneration process [14].

BMPs and TGF-βs are known as the most potent osteogenic growth factor that activate the BMP/TGF-β (transforming growth factor beta) signaling pathway [20, 21]. In this BMP/TGF-β pathway, many intracellular signaling molecules are involved, amongst which Smads, which transmit the extracellular signal through the cytoplasm into the nucleus. The BMP activated signal initiates phosphorylation of Smads1/5/8, while the TGF-β activated signal initiates phosphorylation of Smad2/3 [22]. Phosphorylated Smads subsequently form a complex with Smad4, which is responsible for signal translocation into the nucleus, where the Smad-complex initiates a transcriptional

response with Runx2 activation [21, 23]. Runx2 is the key transcription factor for transcriptional activation of osteogenic gene expression [24, 25]. In addition, Runx2 is the key regulator of the genes that determine osteoblast phenotype, and for Runx2 activity osterix (transcriptional regulator) plays a crucial role [18, 26].

Alternative signaling is possible via the Wnt pathway, which recently received large attention in the field of bone regenerative research due to several studies that indicated the involvement of this pathway in osteogenic differentiation of MSCs [27, 28]. Wnt signaling increases bone mass via diverse mechanisms, including renewal of stromal cells, stimulation of pre-osteoblast replication, and enhancement of osteoblast activity [29]. So far, 19 Wnt family members have been identified. Wnt1 is one of those family members directly involved in upregulation of Runx2 expression [28] and osteogenesis via the activation of Dkk2 expression [30]. In the cytoplasm, the Wnt-transmitted signal stabilizes β-catenin activity [27, 31]. Subsequently, β-catenin translocates into the nucleus, where it induces osteogenic gene expression via activation of transcription factors, i.e. Runx2 and osterix [32, 33, 34, 35, 36]. Many studies demonstrated that Wnt/β-catenin pathway is important in the context of osteogenic differentiation, as the loss of Wnt/β-catenin signaling leads MSCs differentiation into adipocytes [37, 38].

The culture conditions and nutritional supplement source have direct effect on the osteogenic differentiation of MSCs [39, 40, 41], because these supplements are rich in various growth factors and signaling molecules. The most common nutritional supplement used for *in vitro* cell culture is fetal bovine serum (FBS) because of its availability, acceptable costs, and familiarity. However, FBS is not applicable for subsequent pre-clinical and clinical trials, because of potential immunological issues and pathogen transmission [42, 43]. As an alternative, autologous/allogeneic human serum-derived products (e.g. blood plasma, cord blood serum, and platelet lysate) have been explored [39, 40, 41, 44]. Regarding human platelet lysate (PL), sev-

eral studies have demonstrated its decreasing effects on BM-MSCs osteogenic differentiation compared to FBS-supplemented media [11, 45, 46, 47]. Interestingly, AT-MSCs showed opposite results with low osteogenic differentiation in FBS-supplemented medium and high osteogenic differentiation in PL-supplemented medium [48, 49].

This study aimed to evaluate the gene expression profile of components involved in signaling pathways (i.e. BMP/TGFβ and Wnt pathways) related to osteogenic differentiation of different MSCs (i.e. BM-MSCs and AT-MSCs), which were cultured in a medium with different nutritional supplements (either FBS or PL).

We hypothesized that the osteogenic signaling and gene expression profile is dependent on (i) the origin of the MSCs, and (ii) the nutritional supplements (i.e. FBS or PL), which differently affects cell signaling pathways and related osteogenic differentiation. Experimentally, cell proliferation (DNA content), differentiation (ALP activity) and mineralization (calcium deposition) of both AT-MSCs and BM-MSCs cultured in a medium with either of two different nutritional supplements (i.e. FBS or PL) were analyzed. Additionally, the gene expression of osteogenic signaling molecules from the BMP/TGFβ and Wnt pathways (i.e. BMP2, BMP4, TGF-β1, Smad4, Wnt1, β-catenin, Runx2, and osterix) were analyzed.

3.2 Material & Methods

3.2.1 Isolation, expansion & characterization of the cells

AT-MSCs were isolated from fat tissue of healthy humans. The fat tissue was obtained from the Department of Plastic Surgery (Radboudumc, the Netherlands) after written informed consent. BM-MSCs were isolated from human

iliac bone chips, obtained from patients undergoing maxillofacial surgery at the Department of Oral and Craniofacial Surgery (Radboudumc, the Netherlands) after written informed consent. This study was performed according to the principles of the Declaration of Helsinki. The AT-MSCs and BM-MSCs isolation procedure is described in detail elsewhere [3, 11, 21, 30]. Harvested cells were cultured in corresponding proliferation media consisting of alpha Minimal Essential Medium, (α-MEM; Gibco®, Life Technologies, Grand Island, USA) supplemented with either 5% PL (Sanquin Blood Bank, the Netherlands) [11], or 15% fetal bovine serum (FBS; Lonza, Basel, Switzerland) at 37^{o}C in humid atmosphere with 5% CO2 (the complete composition of proliferation media is given in Table 3.1).

Table 3.1: Composition of the proliferation (PM) and osteogenic media (OM)

FBS-supplemented (PM-FBS)	PL-Supplemented (PM-PL)
Minimal Essential Medium (α-MEM) 15% fetal bovin serum (FBS) 0.2 mM L-ascorbic acide 2-phosphate (Vit C) 2 mM L-glutamine 100 U/ml penicillin 10 μg/ ml streptomycin	Minimal Essential Medium (α-MEM) 5% platelet lysate (PL) 10U/ml heparin 100 U/ml penicillin 10 μg/ ml streptomycin
FBS-supplemented (OM-FBS)	PL-Supplemented (OM-PL)
Minimal Essential Medium (α-MEM) 15% fetal bovin serum (FBS) 0.2mM L-ascorbic acide 2-phosphate (Vit C) 2 mM L-glutamine 100 U/ml penicillin 10 μg/ ml streptomycin 10-8 M dexamethasone 0.01 M β-glycerophosphate	Minimal Essential Medium (α-MEM) 5% platelet lysate (PL) 0.2 mM L-ascorbic acide 2-phosphate (Vit C) 2 mM L-glutamine 100 U/ml penicillin 10 μg/ ml streptomycin 10-8 M dexamethasone 0.01 M β-glycerophosphate 0.02 10U/ml heparin

Medium was changed twice a week. Cells were passaged upon reaching 80% confluency using 0.25% w/v trypsin/0.02% EDTA (Gibco®). Cells were examined by fluorescence-activated cell sorting (FACS) for positive expression of CD73, CD90 and CD105 (eBioscience, San Diego, USA) and negative expression of CD45 (R&D system, Abingdon, United Kingdom).

3.2.2 Experimental groups & cell seeding

AT-MSCs and BM-MSCs (passage 4) were cultured in either PL- or FBS-supplemented osteogenic media for 28 days (Table 3.1). The samples for biochemical assays were prepared with 5,000 cells/cm^2 seeding density (n=3). The samples for RNA extraction/Q-PCR analysis were prepared with 10,000 cells/cm^2 seeding density (n=3). The culture medium was changed twice a week and samples were collected at 7 day intervals (days 7, 14, 21 & 28). During the entire culture period, cell morphology was monitored via an inverted light microscope (Leica DM-IL, 5W LED illumination, Rijswijk, the Netherlands).

3.2.3 Cell behavior

Sample preparation for biochemical assays, to determine cell behavior in terms of cellular DNA content, alkaline phosphatase (ALP) activity and calcium deposition, was performed by washing the cell layer twice with PBS, adding 1 ml MilliQ, repetitive freeze/thaw cycles (two times), and finally storage at -80^{o}C till analysis of the DNA and ALP content. Afterwards, the wells were incubated overnight at room temperature with 1 ml 0.5N acetic acid for analysis of Ca deposition.

3.2.3.1 Cellular DNA content

Cellular DNA content was measured using the Quant-iT^{TM} PicoGreen$^{®}$ dsDNA assay kit (Invitrogen; Breda, the Netherlands) according to the manufacturer's instruction. For the standard curve, serial dilutions of dsDNA stock (range: 0-2000 ng/ml) were prepared. Next, 100 μl of either sample or standard solution was added into the wells, followed by 100 μl of working solution. The plate was incubated at room temperature for 5 min, and then the fluorescence of samples/standards was measured with acceptable wavelength range, (excitation at 475-505 nm and emission at 520-550 nm) using a fluorescence microplate reader (FL600, BioTek, Canada).

3.2.3.2 Alkaline phosphatase (ALP) activity

ALP activity was measured using a 5 nM p-nitrophenyl phosphate (4-NP) colorimetric assay. According to the manufacturer's instruction (Sigma-Aldrich, St. Louis, MO, USA), 80 μl of sample solution was combined with 20 μl of buffer (0.5 M 2-amino-2methyl-1-propanol). For the standard curve, serial dilutions of 4-NP (range: 0-25 nmol) were used. Next, 100 μl substrate solution (5 nM p-nitrophenyl phosphate) was added to the samples/standards and incubated for 60 min at 37^{o}C. The reaction was stopped by adding in each well 50 μl 0.3M NaOH. The absorbance of samples was measured at 405 nm using an ELISA microplate reader (EL800, BioTek, Abcoude, the Netherlands). The ALP content was normalized using corresponding cellular dsDNA amount.

3.2.3.3 Calcium deposition

Calcium deposition was measured using the orthocresolphtalein complexone assay (OCPC; Sigma Aldrich, St. Louis, MO, USA), which is based on

a colorimetric reaction between o-cresolphthalein complexone and calcium. According to the manufacturer's instruction, serial dilutions of calcium stock (CaCl2) were prepared (range: 0-100 mg/ml) for the standard curve. For the assay, 10 μl of sample or standard was used, to which 300 μl OCPC solution was added to complete the reaction. After the incubation of plate for 10 min at room temperature, the absorbance was measured at 570 nm using an ELISA microplate reader (EL800, BioTek, Canada).

3.2.4 Gene expression

3.2.4.1 RNA isolation & reverse transcription

To analyze the gene expression profile of selected molecules, RNA was isolated using Genelute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich). According to the manufacturer's instruction, the samples (days 0, 7, 14, 21 and 28) were collected by adding 500 μl 'RNA' lysis buffer and subsequent storage at -80^{o}C. After extraction, the RNA was quantified using a spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). For the reverse transcription (cDNA synthesis), an iScript^{TM} cDNA kit was used (Bio-Rad, Hercules, CA, USA), and for each cDNA reaction 1 μg of mRNA was used. The samples were stored at -20^{o}C until use.

3.2.4.2 Real-Time Polymerase Chain Reaction

For real-time polymerase chain reaction (RT-PCR), qPCR Master Mix Plus /SYBR Green I (Eurogentec; Seraing, Belgium)was used according to the manufacturer's instruction. For each reaction, 1 μg cDNA was used. RT-PCR was completed with 40 amplification cycles. The sequence of applied primers is given in Table 3.2. The raw data were normalized to the expression of RPLP0 (housekeeping gene) within the same sample/mRNA. The gene

expression and fold changes were calculated according to Livak & Schmittgen ($2^{-\Delta\Delta Ct}$) method, relative to BM-PL at the day 0 (50).

Table 3.2: Primer sequences

Gene name	Sequences
RPLP0	Forward- TTCTTCTTTGGGCTGGTCAT Reverse- TTGGGTAGCCAATCTGCAGA
RUNX2	Forward- TCTGGCCTTCCACTCTCAGT Reverse- GACTGGCGGGGTGTAAGTAA
BMP-2	Forward- CCCAGCGTGAAAAGAGAGAC Reverse- GAGACCGCAGTCCGTCTAAG
Wnt-1	Forward- TTCTCCGGGTCCTCCTAAGT Reverse- ATGGCTCCACGACAGAGACT
β-catenin	Forward- GAAACGGCTTTCAGTTGAGC Reverse- CTGGCCATATCCACCAGAGT
TGF-β1	Forward- CAATTCCTGGCGATACCTCAG Reverse- GCACAACTCCGGTGACATCAA
Osterix	Forward- CCTCTGCGGGACTCAACAAC Reverse- AGCCCATTAGTGCTTGTAAAGG
Smad4	Forward- CTCATGTGATCTATGCCCGTC Reverse- AGGTGATACAACTCGTTCGTAGT
BMP4	Forward- TAGCAAGAGTGCCGTCATTCC Reverse- GCGCTCAGGATACTCAAGACC

3.2.5 Statistical analysis

Data are presented as mean ± standard deviation. Statistical analysis was performed based on N=3 (for all experimental groups) with Graphpad Prism ®5.03 software (Graphpad Software Inc, San Diego, CA, USA). Quantitative results were analyzed using a one-way ANOVA with a posthoc Dunnett test (control - BM-PL at day 0). Differences were considered as significant at the $p< 0.05$ value. All experimental cultures were repeated three times.

3.3 Results

3.3.1 Characterization & morphology of MSCs

The results of FACS analysis showed that both cell types (AT-MSCs and BM-MSCs) were 99% positive for expression of surface markers CD73, CD90 and CD105, whereas both these cell types were completely negative for CD45 expression (data not shown).

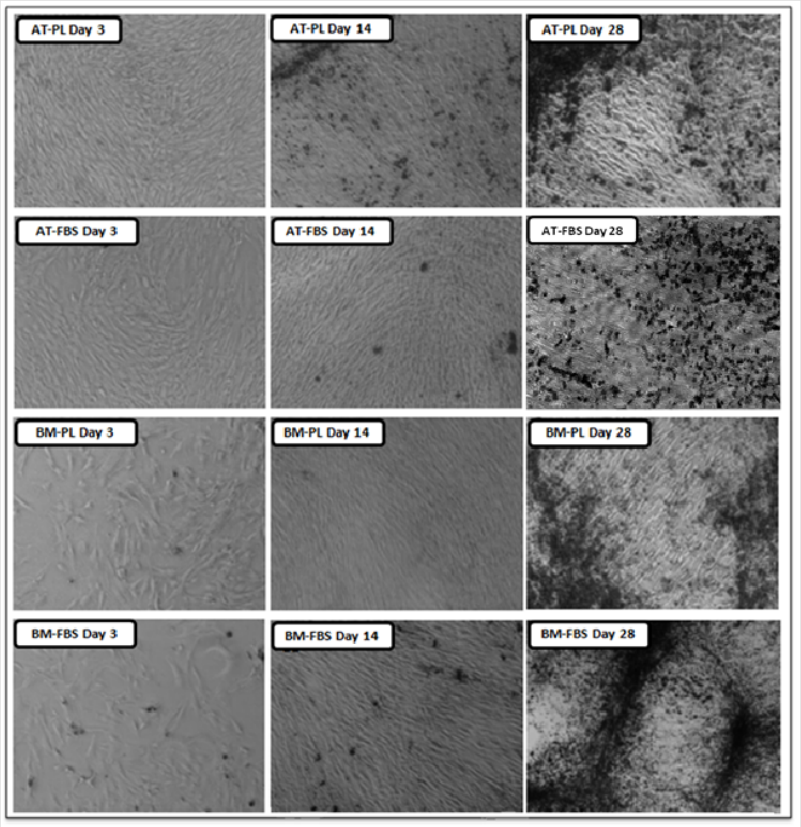

Figure 3.1: Cell morphology monitored with inverted light microscopy, at selected intervals time points, for AT-MSCs and BM-MSCs cultured in PL- or FBS-supplemented media. Black colorization indicates mineralization.

Light microscopy analyses showed that both AT-MSCs and BM-MSCs had an

elongated, spindle-shaped morphology in osteogenic culture condition (Figure 3.1), but after 3 days of culture AT-MSCs appeared smaller with higher cell density than BM-MSCs. After 14 days of culture period, mineralization of the extracellular matrix (black spots) was clearly observed for AT-PL and BM-FBS, and to a lesser extent for BM-PL. After 28 days of culture, substantial mineralization was observed for all experimental groups, except for AT-FBS.

3.3.2 Cell behavior

3.3.2.1 Cellular DNA content

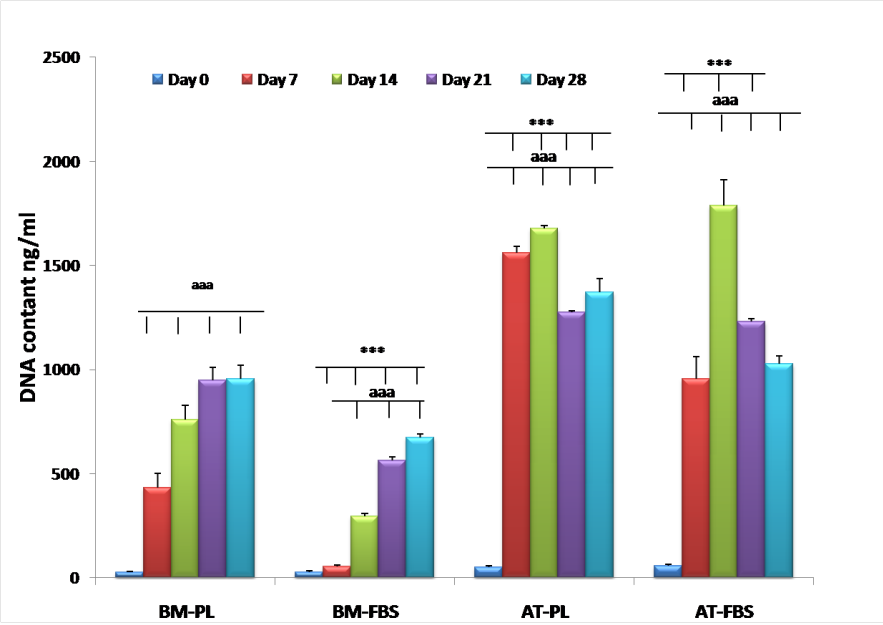

Figure 3.2: Cellular DNA contant of BM-MSCs and AT-MSCs under different culture conditions. The "a" indicates significantly different compared to day 0 (p-values: a $p<0.05$, aa $p<0.01$, aaa $p<0.001$). The "*" indicates significantly different compared to BM-PL value at the same time point (p-values: **$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.)

Analysis of DNA content (Figure 3.2) showed a gradual increase of cell proliferation until day 28 for BM-PL and BM-FBS.The proliferation pattern of AT-PL and AT-FBS was different, with a maximum proliferation at day 14. The level of proliferation of AT-PL ($p< 0.001$) and AT-FBS ($p< 0.001$) was significantly higher compared to both BM-PL and BM-FBS.

3.3.2.2 ALP activity

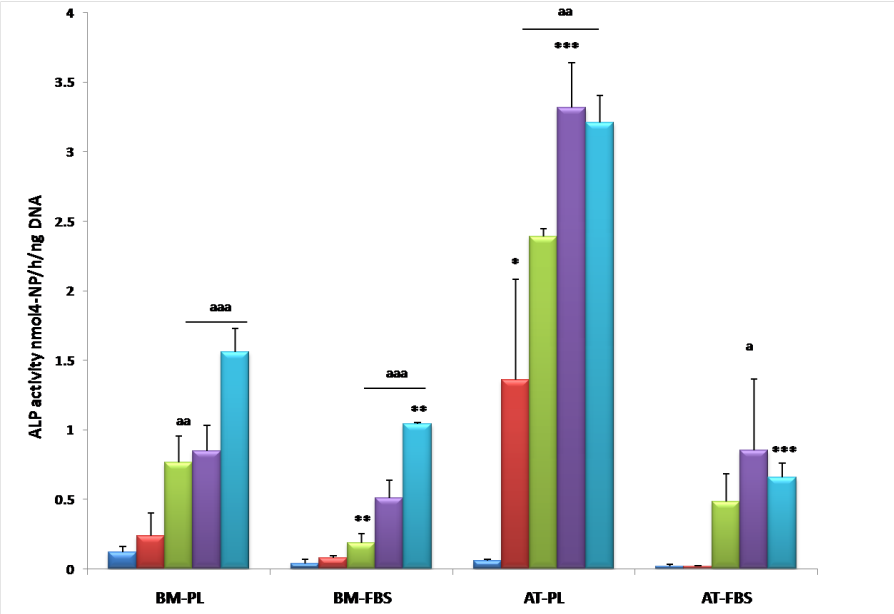

Figure 3.3: ALP activity, of BM-MSCs and AT-MSCs under different culture conditions. The "a" indicates significantly different compared to day 0 (p-values: a $p<0.05$, aa $p<0.01$, aaa $p<0.001$). The "*" indicates significantly different compared to BM-PL value at the same time point (p-values: **$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.)

For both cell types, ALP-activity (early marker for osteogenic differentiation) was high when cells were cultured in PL-supplemented medium (Figure 3.3). With culture time till day 28, a significant increase of ALP-activity was observed for BM-PL and BM-FBS relative to day 0 ($p< 0.001$). In contrast to BM-PL and BM-FBS, the ALP-activity peak for AT-PL and AT-FBS was

at day 21, followed by a decrease till day 28. In AT-PL and AT-FBS, the ALP-activity was significantly increased at all time points relative to day 0 ($p< 0.05$).

3.3.2.3 Calcium deposition

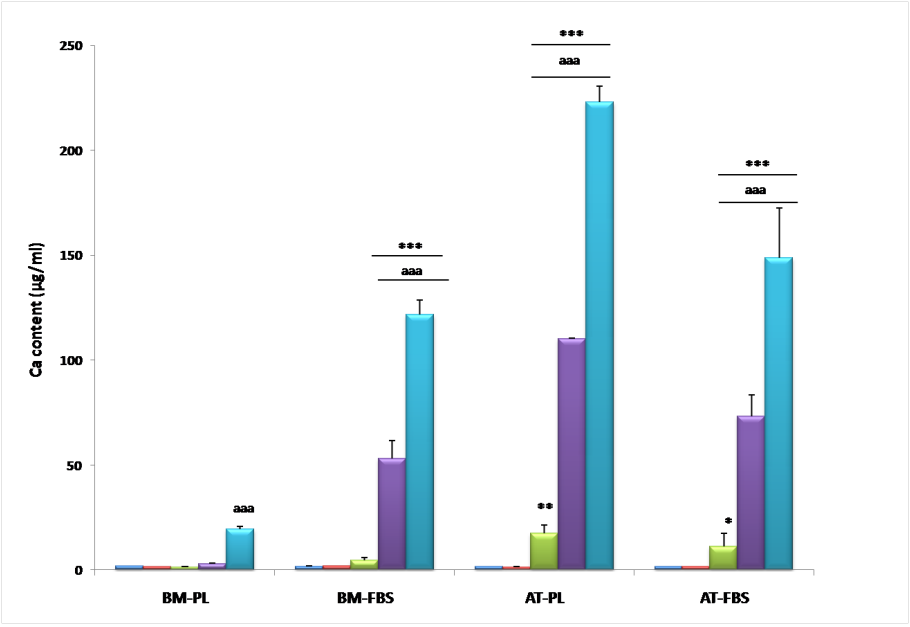

Figure 3.4: Ca deposition in BM-MSCs and AT-MSCs under different culture conditions. The "a" indicates significantly different compared to day 0 (p-values: a $p<0.05$, aa $p<0.01$, aaa $p<0.001$). The "*" indicates significantly different compared to BM-PL value at the same time point (p-values: **$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.)

The results of cellular mineralization, measured by Ca deposition, showed significant differences for cell types as well as for nutritional supplement types (Figure 3.4). Both cell types showed highest mineralization at day 28, but AT-MSCs started to mineralize already from day 14 (in both PL and FBS condition), whereas for BM-MSCs mineralization started from day 21. Moreover, BM-MSCs showed high mineralization in FBS-supplemented and AT-MSCs in PL-supplemented cultures. Significantly increased mineraliza-

tion was observed (relative to day 0 in the same group) for BM-PL at day 28 ($p< 0.001$), for BM-FBS at day 21 and 28 ($p< 0.001$), for AT-PL at days 21 and 28 ($p< 0.001$), and for AT-FBS at days 21 and 28 ($p< 0.001$). Moreover, BM-MSCs showed high mineralization in FBS-supplemented and AT-MSCs in PL-supplemented cultures. Significantly increased mineralization was observed (relative to day 0 in the same group) for BM-PL at day 28 ($p< 0.001$), for BM-FBS at day 21 and 28 ($p< 0.001$), for AT-PL at days 21 and 28 ($p< 0.001$), and for AT-FBS at days 21 and 28 ($p< 0.001$).

3.3.3 Gene expression profile of selected genes

To determine gene expression profiles of selected genes, RT-PCR analysis was performed. The results for each experimental group are described separately.

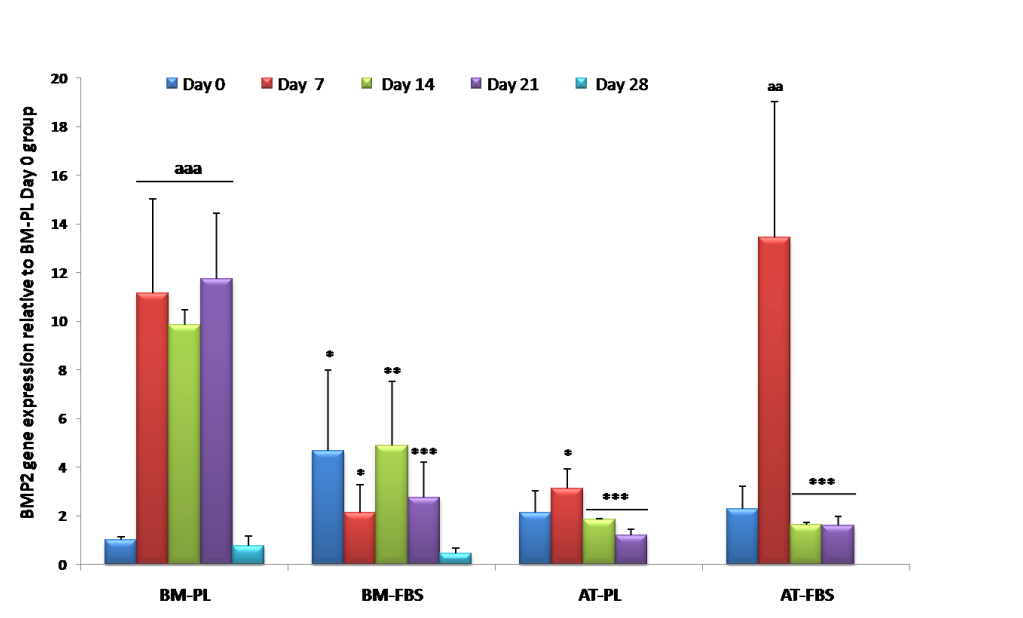

Figure 3.5: Gene expression profile of BMP2. The "a" indicates significantly different compared to day 0 (p-values: a $p<0.05$, aa $p<0.01$, aaa $p<0.001$). The "*" indicates significantly different compared to BM-PL value at the same time point (p-values: **$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.)

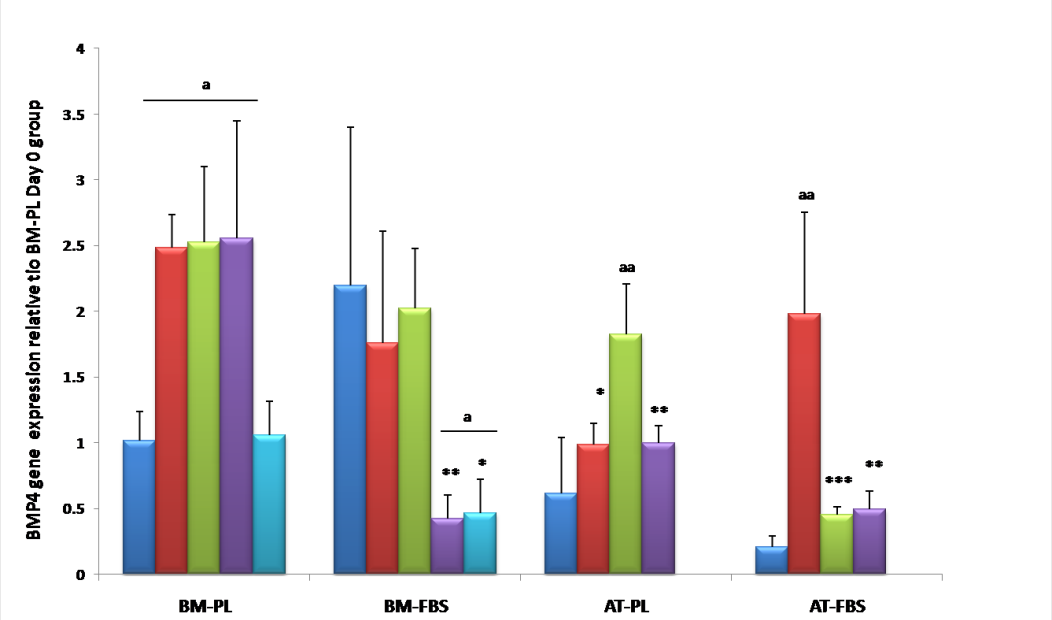

Figure 3.6: Gene expression profile of BMP4. The "a" indicates significantly different compared to day 0 (p-values: a $p<0.05$, aa $p<0.01$, aaa $p<0.001$). The "*" indicates significantly different compared to BM-PL value at the same time point (p-values: **$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.)

BM-PL: BM-PL showed relatively high gene expression levels for the molecules BMP2 and BMP4, as well as for the transcription factors Runx2 and osterix. BMP2 showed an >10-fold upregulation, starting from the first week until third week of culture (Figure 3.5). Similarly, gene expression of BMP4 was upregulated up to 2.5-fold (Figure 3.6). The expression profiles of BMP2 and BMP4 were similar, but the amount of BMP4 was relatively low compared to BMP2. In contrast to BMPs, the expression of TGF-β1 was downregulated over the entire culture period, except for day 7 (Figure 3.7). Smad4 expression was upregulated especially at 14 days of culture (Figure 3.8). Wnt1 was upregulated during the first 21 days of culture. The expression pattern of Wnt1 was similar to BMP2 and BMP4 expression (Figure 3.9). At the same time β-catenin (from Wnt pathway) was downregulated (Figure 3.10).

Finally, gene expression of transcription factors Runx2 and osterix was up-regulated during first three weeks of culture (Figure 3.9), Figure 3.10).

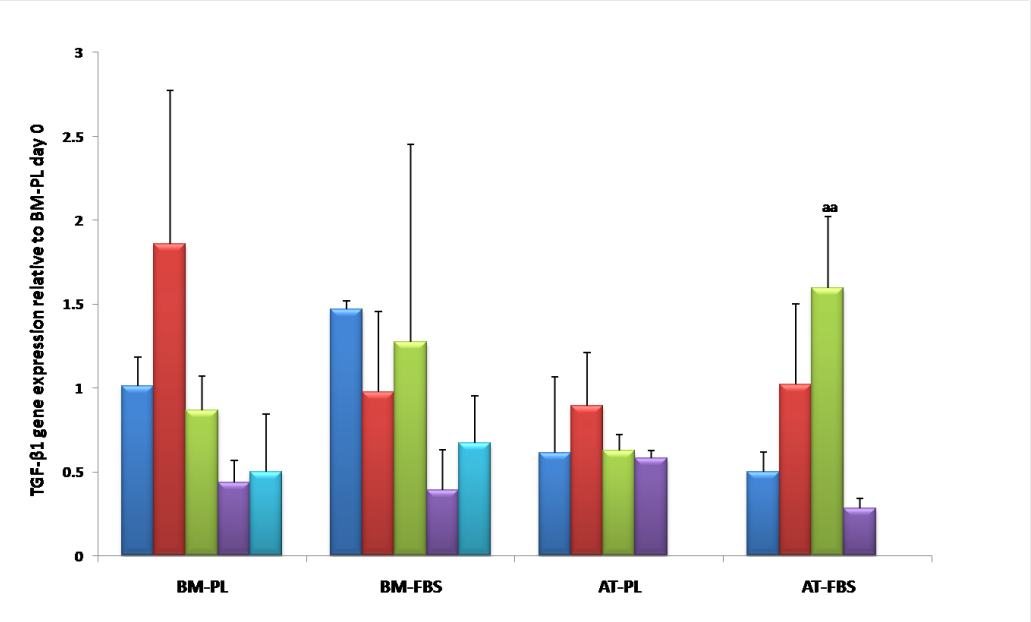

Figure 3.7: Gene expression profile of TGFβ. The "a" indicates significantly different compared to day 0 (p-values: a $p<0.05$, aa $p<0.01$, aaa $p<0.001$). The "*" indicates significantly different compared to BM-PL value at the same time point (p-values: **$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.)

BM-FBS: For BM-FBS, BMP2 expression was relatively high (4.5 fold) already in proliferation media (day 0). In osteogenic conditions, the expression of BMP2 was not stable, showing variations during 28 days of culture period (Figure 3.5). BMP4 expression was upregulated (up to 2.5 fold) during the first two weeks of culture, and afterwards it was downregulated until day 28 (Figure 3.6). During the entire culture period, TGF-β1 expression was downregulated (Figure 3.7). For BM-FBS, the expression pattern of Smad4 was similar to BM-PL, with an upregulation (up to 6.5 fold) in the second week of culture (Figure 3.8).

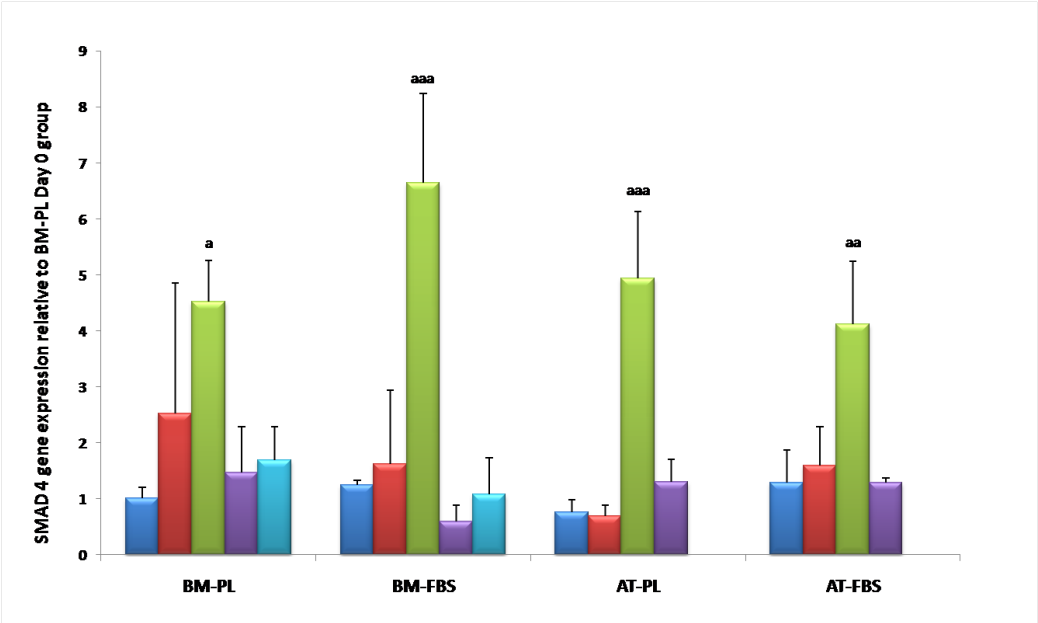

Figure 3.8: Gene expression profile of Smad4. The "a" indicates significantly different compared to day 0 (p-values: a $p<0.05$, aa $p<0.01$, aaa $p<0.001$). The "*" indicates significantly different compared to BM-PL value at the same time point (p-values: **$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.)

The expression of Wnt1 was upregulated (up to 5.5 fold) in the second and third weeks (Figure 3.9). The expression of β-catenin was downregulated during the entire culture period (Figure 3.10). For osteogenic transcription factors, an upregulation of Runx2 (up to 2 fold) was observed in the first, second and fourth week of culture (Figure 3.11). For BM-FBS, the level of Runx2 was substantially lower than for BM-PL. Finally, an upregulation of osterix was observed already in proliferation media, but after the first week of its gene expression was downregulated, and on day 28 no expression of osterix was detectable anymore (Figure 3.12).

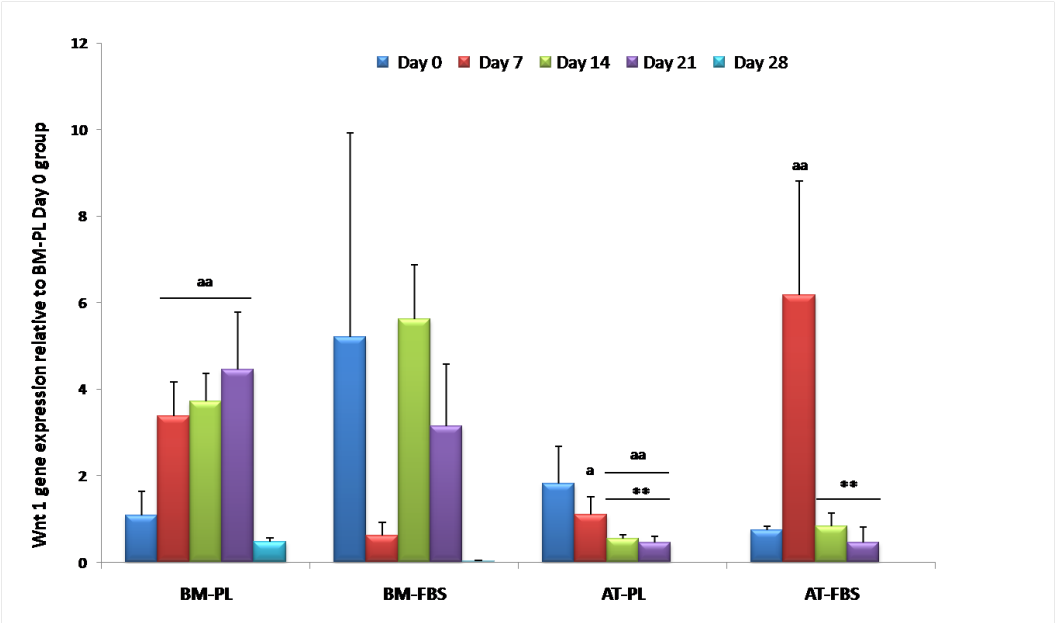

Figure 3.9: Gene expression profile of Wnt1. The "a" indicates significantly different compared to day 0 (p-values: a $p<0.05$, aa $p<0.01$, aaa $p<0.001$). The "*" indicates significantly different compared to BM-PL value at the same time point (p-values: **$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.)

AT-PL: In contrast to BM-PL and BM-FBS, the expression of BMP2 was very low (up to 3 fold) in AT-PL. A small upregulation of BMP2 was observed only in the first week of culture (Figure 3.5). BMP4 was upregulated (up to 1.7 fold) in the second week of culture (Figure 3.6). TGF-β1 was downregulated during the entire culture period (Figure 3.7). An upregulation of Smad4 (up to 6.5 fold) was detected again in the second week of culture (Figure 3.8). In contrast to BM-PL and BM-FBS, for AT-PL the expression of Wnt1 and β-catenin was downregulated during the entire culture time (Figure 3.9), (Figure 3.10) but in proliferation media (day 0) the level of β-catenin was relatively high (up to 1.5 fold). For transcription factors, Runx2 was upregulated (up to 5 fold) in the second and third week of culture (Figure 3.11), and osterix was downregulated during the entire culture

period (Figure 3.12).

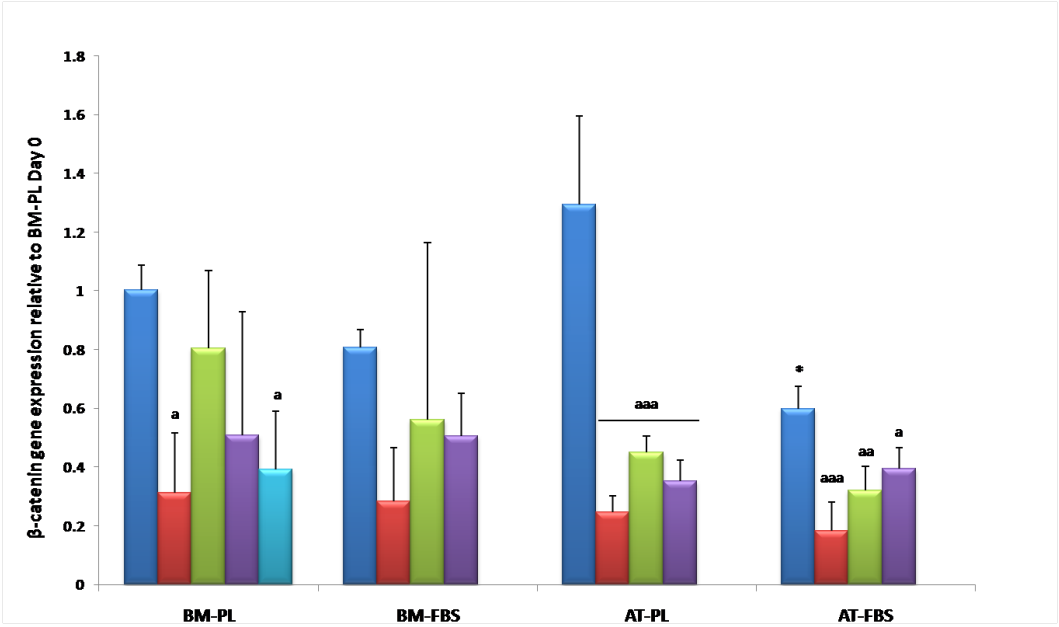

Figure 3.10: Gene expression profile of β-catenin. The "a" indicates significantly different compared to day 0 (p-values: a $p<0.05$, aa $p<0.01$, aaa $p<0.001$). The "*" indicates significantly different compared to BM-PL value at the same time point (p-values: **$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.)

AT-FBS: The expression of BMP2 for AT-FBS was upregulated (up to 13 fold) in the first week of culture (Figure 3.5); afterwards it decreased to baseline values. For AT-FBS, the expression of BMP4, Wnt-1 and osterix was similar to BMP2 showing an upregulation only at day 7 (Figure 3.6, Figure 3.9, Figure 3.10). TGF-β1 was upregulated (up to 1.7 fold) in the second week of culture (Figure 3.7). An upregulation of Smad4 (up to 4 fold) was observed again in the second week of culture (Figure 3.8). The expression of β-catenin was downregulated during the entire culture period (Figure 3.10). For transcription factors, Runx2 was upregulated in the second and third weeks of culture and the expression pattern of osterix was similar to BMP2, showing an upregulation in the first week of culture (Figure 3.11, 3.12).

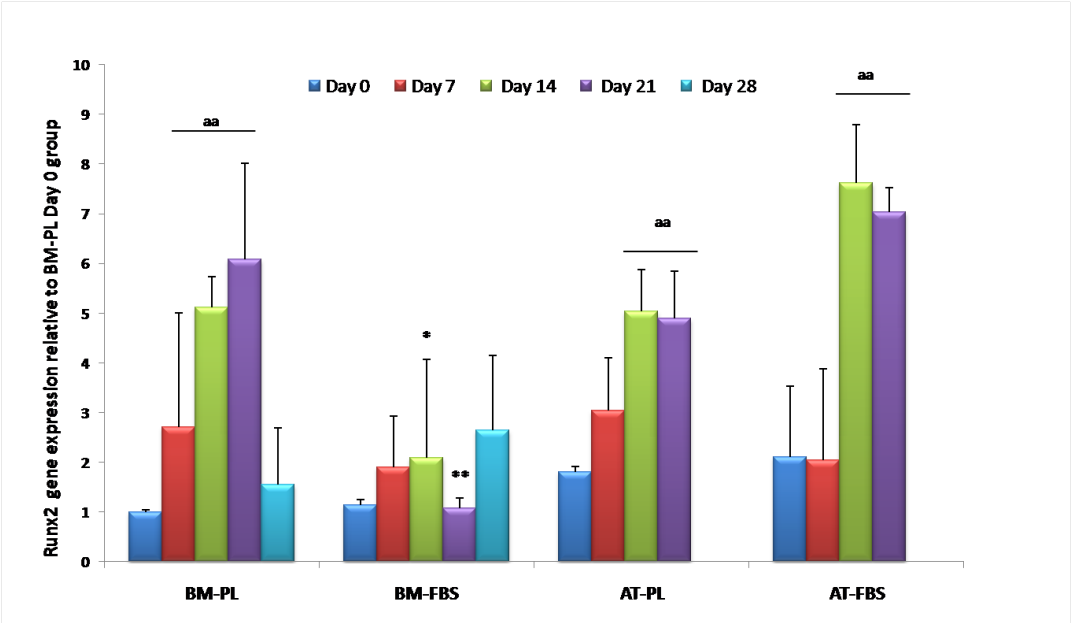

Figure 3.11: Gene expression profile of RUNX2. The "a" indicates significantly different compared to day 0 (p-values: a $p<0.05$, aa $p<0.01$, aaa $p<0.001$). The "*" indicates significantly different compared to BM-PL value at the same time point (p-values: **$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.)

3.4 Discussion

This study aimed to evaluate the gene expression profile of components involved in signaling pathways (i.e. BMP/TGFβ and Wnt pathways) related to osteogenic differentiation of different MSCs (i.e. BM-MSCs and AT-MSCs), which were cultured in a medium with different nutritional supplements (either FBS or PL).

We hypothesized that the osteogenic signaling and gene expression profile is dependent on (i) the origin of the MSCs, and (ii) the nutritional supplements (i.e. FBS or PL), which differently affect cell signaling pathways and related osteogenic differentiation. The main findings of this study showed that BM-MSCs show a more pronounced mineralization in FBS-supplemented media,

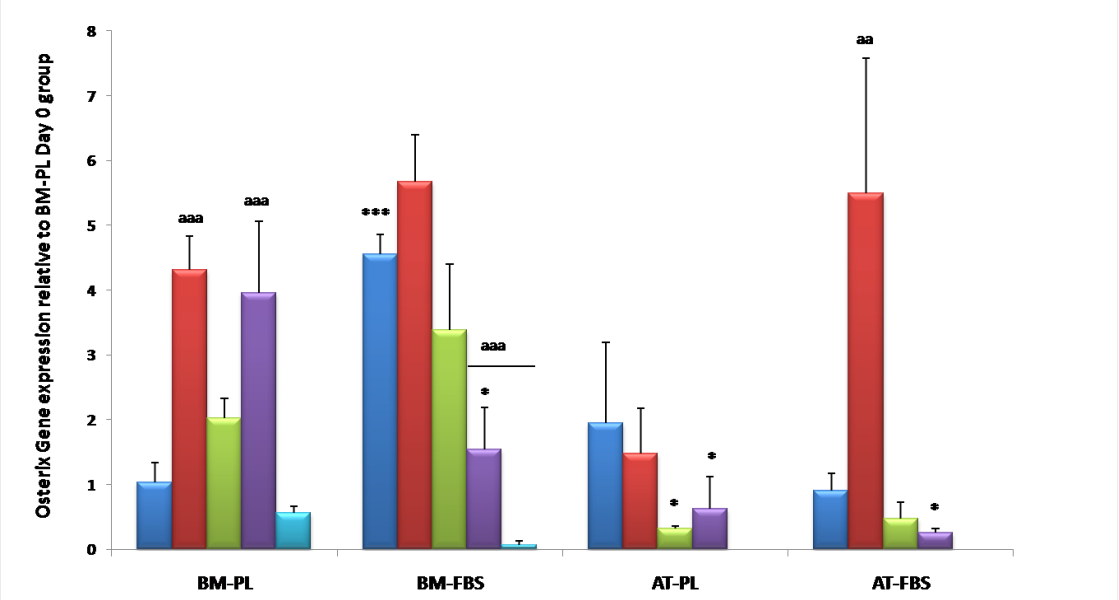

Figure 3.12: Gene expression profile of Osterix. The "a" indicates significantly different compared to day 0 (p-values: a $p<0.05$, aa $p<0.01$, aaa $p<0.001$). The "*" indicates significantly different compared to BM-PL value at the same time point (p-values: **$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.)

while AT-MSCs do so in PL-supplemented media. Remarkably, BM-MSCs in PL-supplemented media highly express several osteogenic genes (i.e. BMP2, BMP4, Wnt1, Runx2, and osterix) in contrast to AT-MSCs, which only highly express Runx2. Furthermore, a high level of parallelism in BMP2, BMP4 and Wnt1 gene expression was observed, irrespective of MSC origin or nutritional supplement.

The data of biochemical assays showed remarkable differences between cell types and supplement types. Both cell types showed high proliferation (DNA content) and differentiation (ALP activity) capacity in PL-supplemented media. However, calcium deposition showed opposite results, with for BM-MSCs highest mineralization in FBS-supplemented media, while for AT-MSCs in PL-supplemented media. A similar observation was recently reported in [11]. This observation indicates that although BM-MSCs and AT-

MSCs are morphologically similar, the gene expression profiles of several osteogenic genes are different, which differently affect osteogenic differentiation of cells.

BM-MSCs in PL-supplemented culture highly expressed the most potent osteogenic molecules i.e. BMP2, BMP4, Wnt1, Runx2 and osterix. However, the mineralization pattern of BM-PL did not correlate with the level of the aforementioned gene expression profiles. An upregulation of BMP2 and ALP expression by BM-MSCs cultured in PL-supplemented media was reported previously [45]. These data suggest that BMP2, BMP4, Wnt1, Runx2 and osterix have high potential to be involved in early stage differentiation of BM-PL. However, at later stages of osteogenic differentiation of BM-PL, those molecules seem not to be crucial. Moreover, gene expression profiles for BM-MSCs in FBS-supplemented media also showed an upregulation of BMP2, BMP4, Wnt1, Runx2 and osterix, which correlated with high levels of mineralization. These results confirm our hypothesis that osteogenic gene expression profiles are dependent on the nutritional supplement (i.e. FBS or PL), which differently affects cell signaling pathways and related osteogenic differentiation.

The molecular mechanisms of proliferation and osteogenic differentiation of AT-MSCs are different from those of BM-MSCs, because the gene expression of most osteogenic molecules (i.e. BMP2, BMP4, Wnt1, osterix; not Runx2) was downregulated in AT-MSCs or expressed at rather low levels. These remarkable differences in expression levels of osteogenic signaling molecules between these two cell types confirm our hypothesis that the gene expression profile is dependent on the origin of the MSCs. Runx2 was one of few molecules, which was upregulated for AT-MSCs, in both PL- and FBS- supplemented cultures. Interestingly, AT-MSCs showed high levels of calcium deposition (for both AT-PL and AT-FBS conditions), which is an indication about a possible correlation between Runx2 expression and AT-MSC mineralization. It is known that calcium can accumulate in intracellular stores,

mainly via calcium channels by help of Runx2 [50], which could be linked to the high mineralization of AT-MSCs. Osterix is considered as a downstream regulator of Runx2 [24]. However, in our observations, the expression of osterix was not in accordance with Runx2; osterix expression profiles were rather similar to those of BMP2 and BMP4, which implies that BMP expression is osterix-dependent or BMP-signaling induces the osterix expression [2].

Interestingly, Wnt1 expression profiles were also similar to BMP2 and BMP4 in each separate group. It seems that BMPs control Wnt1 expression or Wnt1 induces BMP expression. A recent report about the correlation of BMP2 and Wnt signaling showed that BMP2-mediated extracellular matrix mineralization is involved in activation of Wnt signaling [51]. To fully elucidate a possible correlation between Wnt signaling and mineralization of MSCs (AT-MSCs or BM-MSCs), more genes from the Wnt pathway need to be analyzed.

The results of gene expression of TGF-β1, Smad4 and β-catenin indicate that these molecules are not directly involved in osteogenic differentiation of MSCs, as TGF-β1 and β-catenin were downregulated at almost all culture periods for both cell types. An upregulation of Smad4 was detected only in the second week of culture for all experimental groups, which afterwards decreased back to baseline values again. This observation suggests that the expression of Smad4 is not cell type-specific or supplement-specific, because the expression profile for all experimental groups was similar. However, Smad4 is known to be a crucial element for the transmission of BMP- as well as TGF-β-activated signals into the nucleus [52]. An upregulation of Smad4 in the second week could indicate its temporary involvement in turning on during osteogenic differentiation.

The phenomenon of maximized mineralization capacity of AT-MSCs in PL-supplemented and BM-MSCs in FBS-supplemented media still remains elusive. PL contains many signaling molecules (growth factors), which can acti-

vate osteogenic signaling mechanisms for AT-MSCs, but less for BM-MSCs. Possible explanations for this phenomenon include the concentration of PL in the media (5%), which was probably sufficient to promote BM-MSCs proliferation, but not mineralization. It is known that an appropriate dosage of proteins is crucial for normal bone development [24], and overexpression of osteogenic proteins may cause unexpected disorders [52].

The fact that AT-MSCs have high osteogenic activity in PL-supplemented media is appealing for their use in subsequent (pre-)clinical applications. Together with the rapid proliferation and the knowledge that *in vitro* expansion of MSCs requires a safe nutritional component in the culture medium (e.g. autologous PL), AT-MSCs seem more appropriate for this purpose than BM-MSCs. Comparative evaluations of both cell types regarding their (pre-) clinical efficacy are required to address this issue.

3.5 Conclusions

Our data showed that the cellular behavior and molecular mechanisms of osteogenic differentiation of BM-MSCs and AT-MSCs are rather different, which confirms dependency of the osteogenic gene expression profile on the origin of the MSCs. Different nutritional supplements stimulated alternative signaling pathways for controlling the proliferation and osteogenic differentiation of BM-MSCs and AT-MSCs. BMP/TGF-β and Wnt signaling seem to be involved in proliferation and early differentiation of BM-MSCs, whereas Runx2 is potentially involved in osteogenic differentiation and mineralization of AT-MSCs. These observations confirm that osteogenic gene expression profiles are dependent on the nutritional supplement (i.e. FBS or PL), which differently affects cell signaling pathways and related osteogenic differentiation. The fact that AT-MSCs showed rapid proliferation and high mineralization capacity, especially in PL-supplemented media, is appealing

for their application in subsequent (pre-) clinical research, as an alternative cell source for BM-MSCs.

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CHAPTER 4

Osteogenic capacity of human adipose tissue-derived mesenchymal stromal cells in co-culture with endothelial cells or macrophages

4.1 Introduction

One of the challenges confronted by orthopedic surgeons is to repair large segmental bone defects caused by trauma, infection, tumor resection, skeletal bone abnormalities or other bone disorders. So far, such clinical challenges have been predominantly approached using autologous bone grafts. However, this approach is accompanied by multiple limitations, e.g. quantity and quality issues of autologous donor bone and issues related to a second surgery site [1]. Alternative treatment options have been explored using the toolbox of tissue engineering (i.e. scaffolds, biologicals and cells). In view of cell-based strategies, major emphasis has focused on mesenchymal stromal cells (MSCs) [2]. These MSCs can be obtained from various tissues, including bone marrow (BM), skin, muscle and adipose tissue (AT). However, the yield of MSCs

usually varies within these different tissues [3]. In contrast to other donor tissues, substantial numbers of MSCs can be harvested from AT, with less morbidity and easy accessibility compared to other tissue sources [4]. Moreover, AT-MSCs have shown high potential for osteogenic differentiation and mineralization in vitro [4, 5, 6].

To develop cell-based bone tissue regenerative strategies, it is necessary to understand natural bone regeneration and fracture healing processes. Bone is a highly vascularized tissue with self healing, regeneration and remodeling ability, representing biological processes that involve many cell types [7]. Vascularization is an essential process during bone fracture healing, regeneration and remodeling. Fracture healing starts with an inflammatory phase, followed by reparative and remodeling phases. During inflammation, white blood cells (i.e. monocytes, macrophages, neutrophils, and lymphocytes) accumulate at the defect site, where they form granulation tissue [8]. Next, MSCs and endothelial cells migrate to the defect site, where they promote vascularization and bone repair. In this phase, many signaling mechanisms evoke subsequent differentiation of MSCs into osteoblast and formation of osteoclasts from hematopoietic precursor cells. Fracture healing is completed with a bone remodeling phase, during which bone retains its original shape, structure, and mechanical strength [8]. Consequently, bone regeneration and fracture healing are complex physiological processes, in which many cell types (i.e. MSCs, endothelial cells, macrophages) and multiple factors (signaling pathways) are involved, which coordinate cell migration, cell proliferation, osteogenic differentiation and extracellular matrix formation [9]. MSCs from different origin co-cultured with endothelial cells already received huge interest in bone regenerative research, and it was proven that endothelial cells promote mineralization of MSCs in vitro and bone formation in vivo [10, 11]. Moreover, cellular crosstalk between MSCs and endothelial cells activates adequate osteogenic signals aided by the expression of signaling factors [12] (e.g. vascular endothelial growth factor; VEGF), which is shown to be essential for bone repair [13]. Several studies indicate that macrophages play a

crucial role in bone regeneration, because they are involved in extracellular matrix formation [14, 15] and secrete a plethora of signaling molecules that are linked to multiple processes during osteogenesis [16, 17]. In view of this, it is straightforward to study the behavior of AT-MSCs in in vitro culture conditions based on co-culture with endothelial cells or macrophages [12].

Different studies, which focused on cell-cell interactions in a direct or indirect co-culture set-up [18, 19, 20] , showed that direct cell-cell contact has different effects on cellular communication compared to indirect contact [21, 22]. This indicates that cell co-culture set-up plays an important role in cellular communication [18]. MSCs in direct contact with endothelial cells showed positive effects regarding osteogenic differentiation in comparison to indirect contact [10]. It has been proven that direct cellular contact between MSCs and adult cardiomyocytes promotes MSC differentiation into cardiomyocytes, in contrast to indirect contact. Moreover, in an indirect co-culture set-up, signaling molecules (growth factors), which were secreted in the culture medium, showed no effect on MSC differentiation [21]. Consequently, it is important to understand what mechanisms are involved in direct and indirect cell-cell communication [23, 24].

This study aimed to evaluate the osteogenic differentiation of AT-MSCs upon co-culture with endothelial cells or macrophages in a direct or indirect co-culture set-up. Our hypotheses were that (i) endothelial cells and macrophages stimulate AT-MSCs proliferation and osteogenic differentiation, and that (ii) these two cell types will more profoundly affect osteogenic differentiation of AT-MSCs in a direct compared to an indirect co-culture set-up, because of the possibility for both cell-cell interactions and effects of secreted soluble factors.

4.2 Materials & Methods

4.2.1 Cell-culture

AT-MSCs were isolated from fat tissue of three different healthy human donors. Fat tissue was obtained from the Department of Plastic Surgery (Radboudumc, Nijmegen, the Netherlands) after written informed consent. This study was performed according to the principles of the Declaration of Helsinki. The procedure of AT-MSCs isolation is described in detail elsewhere [4, 10, 25, 26].

Table 4.1: Composition of the proliferation and osteogenic media

Proliferation Media (PM)	Osteogenic Media (OM)
Minimal Essential Medium (a-MEM)	Minimal Essential Medium (a-MEM)
5% platelet lysate (PL)	5% platelet lysate (PL)
10U/ml heparin	0.2 mM L-ascorbic acide 2-phosphate (Vit C)
100 U/ml penicillin	2 mM L-glutamine
10 μg/ ml streptomycin	100 U/ml penicillin
	10 μg/ ml streptomycin
	10-8 M dexamethasone
	0.01M β-glycerophosphate
	10U/ml heparin

Harvested cells were expanded in proliferation medium, consisting of alpha minimal essential medium, (α-MEM; Gibco®, Life Technologies, Grand Island, USA) supplemented with 5% platelet lysate (PL, Sanquin Blood Bank, Nijmegen, the Netherlands), pooled from 5 different donors, 100 U/ml penicillin (Gibco®), 10 μg/ml streptomycin (Gibco®), and 10 U/ml heparin (LEO Pharma, Amsterdam, the Netherlands) at 37°C in a humid atmosphere with 5% CO2. Medium was changed twice a week. Cells were passaged upon

reaching 80% confluency using 0.25% w/v trypsin/0.02% EDTA (Gibco®) (Table 4.1). AT-MSCs, before using them in experimental work, were examined by fluorescence-activated cell sorting (FACS) for positive expression of stem cell markers CD73, CD90 and CD105 (eBioscience, San Diego, USA) and negative expression of CD45 (R&D system, Abingdon, United Kingdom). All donors were checked via biochemical assays for their osteogenic potential, i.e. ALP activity and calcium deposition.

As endothelial cells, Human Umbilical Vein Endothelial Cells (HUVECs) were obtained from a commercial source (Becton Dickinson Biosciences, BD, Breda, the Netherlands). Cells were expanded at 37°C in proliferation medium consisting of Medium 200 (Gibco®), supplemented with low serum growth supplement kit (LSGS; Gibco®), in humid atmosphere with 5% CO2. Medium was changed twice a week. Cells were passaged upon reaching 80% confluency using 0.25% w/v trypsin/0.02% EDTA.

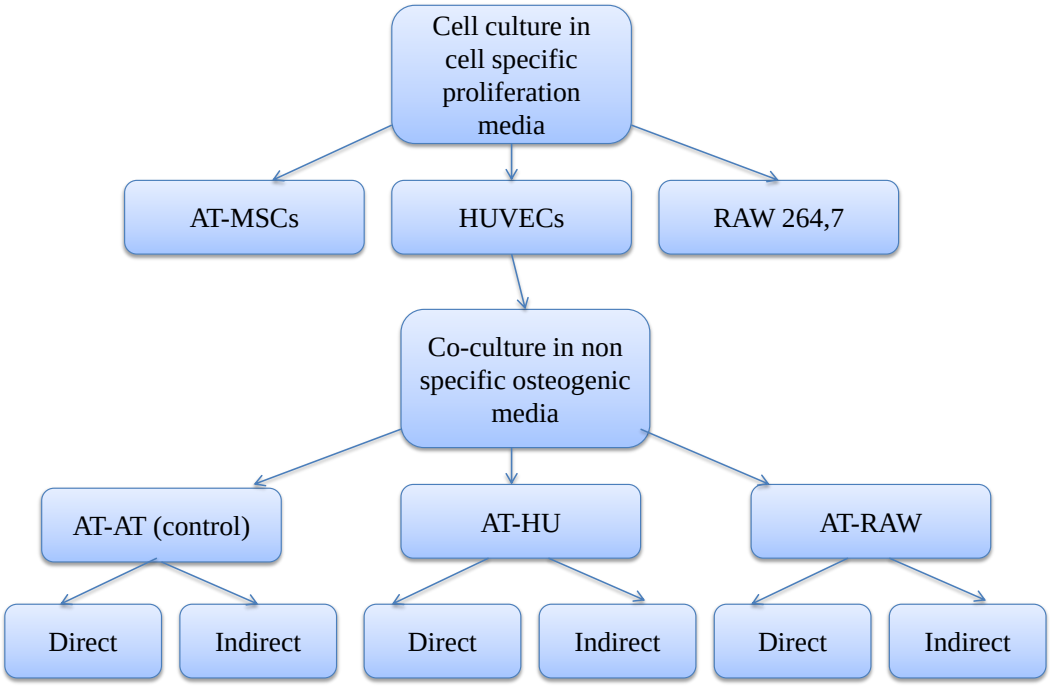
As a monocyte/macrophage cell type, RAW 264.7 cells were used, which were obtained from a commercial source (Sigma-Aldrich, St. Louis, MO, USA). RAW 264.7 cells were expanded in proliferation medium consisting of Dulbecco's Modified Eagle Medium (D-MEM Gibco®), supplemented 2 mM L-glutamine and 10% fetal bovine serum (FBS, Lonza, Basel, Switzerland). Medium was refreshed twice a week and cells were passaged upon reaching 80% confluency.

4.2.2 Experimental co-cultures

For the direct co-culture set-up, AT-MSCs were cultured with either HUVECs or RAW264.7 cells in a 1:1 cell ratio, with total number of 10.000 cells/cm^2 (24 well plates /n=3). As a control, AT-MSC monoculture (10.000 cells/cm^2) was used (Table 4.2). The experimental groups were following: direct AT-AT (AT-MSCs in monoculture as a control), AT-HU (AT-MCSs

co-cultured with HUVECs) and AT-RAW (AT-MCSs co-cultured with Raw 264.7 cells).

Table 4.2: Overview of co-culture systems of AT-MSCs



For the indirect co-culture set-up, AT-MSCs were cultured in 24 well plates, which were combined with transwell inserts (Greiner Bio-One, Kremmnster, Austria), into which HUVECs or RAW264.7 cells were cultured. The cell seeding density in 24 well plates was 10.000 cells/cm^2 (n=3) and in transwells, the same number of cells were seeded to maintain a 1:1 cell ratio. As a control, AT-MSCs were co-cultured with AT-MSCs in transwells. Experimental groups were following: indirect AT-AT (AT-MSCs co-cultured with AT-MSCs), AT-HU and AT-RAW. For all co-culture groups, the same osteogenic medium was used consisting of α-MEM supplemented with 5%

PL, 100 U/ml penicillin, 10 μg/ml streptomycin, 10 U/ml heparin, 0.2 mM L-ascorbic acid-2-phosphate (Sigma-Aldrich, St. Louis, MO, USA), 2mM L-glutamine (Gibco®), 10-8M dexamethasone (Sigma-Aldrich) and 0.01 M β-glycerophosphate (Sigma-Aldrich), at 37°C in a humid atmosphere with 5% CO2 (Table 4.2).

4.2.3 Cell morphology

During the entire culture period, cell morphology was monitored with an inverted light microscope (Leica DM-IL, 5W LED Illumination, Rijswijk, the Netherlands) for both direct and indirect co-cultures. Additionally, cell morphology was monitored with scanning electron microscopy (SEM; JOEL 6330F, Peabody, USA), for which samples were prepared at selected time points by washing twice with PBS, fixing with 2% glutaraldehyde for 10 minutes, and washing with 0.1M Na-cacodylate buffer for 10 minutes. For dehydration, samples were treated with a graded series of ethanol, each for 5 minutes. Finally, samples were incubated in air flow overnight by adding one drop of tetramethylsilan to each sample for complete dehydration. Prior to observation using a SEM, samples were coated with a gold layer.

4.2.4 Cell behavior

Cell behavior was analyzed with different biochemical assays, which were used as described previously [10]. Cellular DNA content was used as a measure for cell proliferation and quantified with the QuantiFluor® dsDNA kit (Promega, Madison, Wisconsin, USA). To determine early osteogenic differentiation, alkaline phosphatase (ALP) activity was measured using a colorimetric assay (Sigma-Aldrich, St. Louis, MO, USA) and normalized to cellular DNA content. Finally, for late stage osteogenic differentiation, calcium deposition was measured using a p-nitrophenyl phosphate (4-NP)

colorimetric assay (Sigma-Aldrich, St. Louis, MO, USA).

Samples from cell culture experiments were taken at the days 7, 14, 21, 28 and 35. For sampling, cell layers were washed twice with PBS, and stored with 1 ml MilliQ at $-80°C$. After two repetitive freeze/thaw cycles, samples were used as per instructions of the manufacturer of the assay kits. Sampling for calcium deposition, measurements was performed by incubating the remaining extracellular matrix in the 24 well plates overnight at room temperature with 1 ml 0.5N acidic acid on a shaking plate. For direct co-culture, the ALP and calcium deposition data were normalized with initial AT-MSCs cell seeding number.

4.2.5 Statistical analysis

The data was statistically analyzed using Graphpad Prism software (GraphPad Software INC.7825 Fay Avenue, Suite 230 La Jolla, CA 92037, USA). An one-way ANOVA with post-hoc Dunett's comparisons test was used to analyze the effect of different cell types on proliferation, differentiation and mineralization. An unpaired t-test was used to analyze the data between different co-culture set-up, i.e. direct versus indirect. Differences were considered as significant at $p < 0.05$.

4.3 Results

This study is based on results using AT-MSCs of three different human donors. All donor cells were first characterized with FACS for expression of stem cell markers (CD73, CD90, and CD105). The results of FACS analysis showed that AT-MSCs (all three donors) were 99% positive for expression of these markers and completely negative for CD45 expression (data not shown). All donor cells further showed positive results for differentiation

and mineralization (based on ALP activity and Ca deposition results). A comparative overview of co-culture results compared to simultaneous respective AT-MSCs mono-culture controls is presented in Table 4.3. Similarly, a comparative overview of direct versus indirect co-culture results is presented in Table 4.4.

Table 4.3: Overview of co-culture results of AT-MSCs from 3 donors compared to respective AT-MSCs mono-cultures.

	AT-HU	AT-HU	AT-RAW	AT-RAW
	Direct	Indirect	Direct	Indirect
Proliferation				
Donor 1	0[1]	0	+	0
Donor 2	0	0	+[2]	0
Donor 3	0	0	+	0
Differentiation				
Donor 1	+	+	-[3]	+
Donor 2	+	±[4]	-	-
Donor 3	+	±	-	-
Mineralization				
Donor 1	+	+	-	+
Donor 2	+	-	-	-
Donor 3	+	0	-	0

[1] no differences in proliferation, differentiation or mineralization of experimental groups compared to AT-MSCs mono-culture

[2] increased proliferation, differentiation or mineralization of experimental groups compared to AT-MSCs mono-culture control

[3]decreased proliferation, differentiation or mineralization of experimental groups compared to AT-MSCs mono-culture control

[4] variable proliferation, differentiation or mineralization in experimental groups compared to AT-MSCs mono-culture control

Table 4.4: Overview of direct versus indirect co-culture results with AT-MSCs from 3 human donors.

	AT-HU	AT-RAW
	Direct vs indirect co-culture	Direct vs indirect co-culture
Proliferation		
Donor 1	0	-
Donor 2	+	-
Donor 3	+	-
Differentiation		
Donor 1	+	+
Donor 2	-	+
Donor 3	-	+
Mineralization		
Donor 1	-	+
Donor 2	-	+
Donor 3	-	+

4.3.1 Cell morphology

With inverted light microscopy, it was possible to monitor the cell morphology of all experimental groups. The results at days 7 and 35 for both direct and indirect culture set-up are presented in (Figure 4.1 and 4.2).

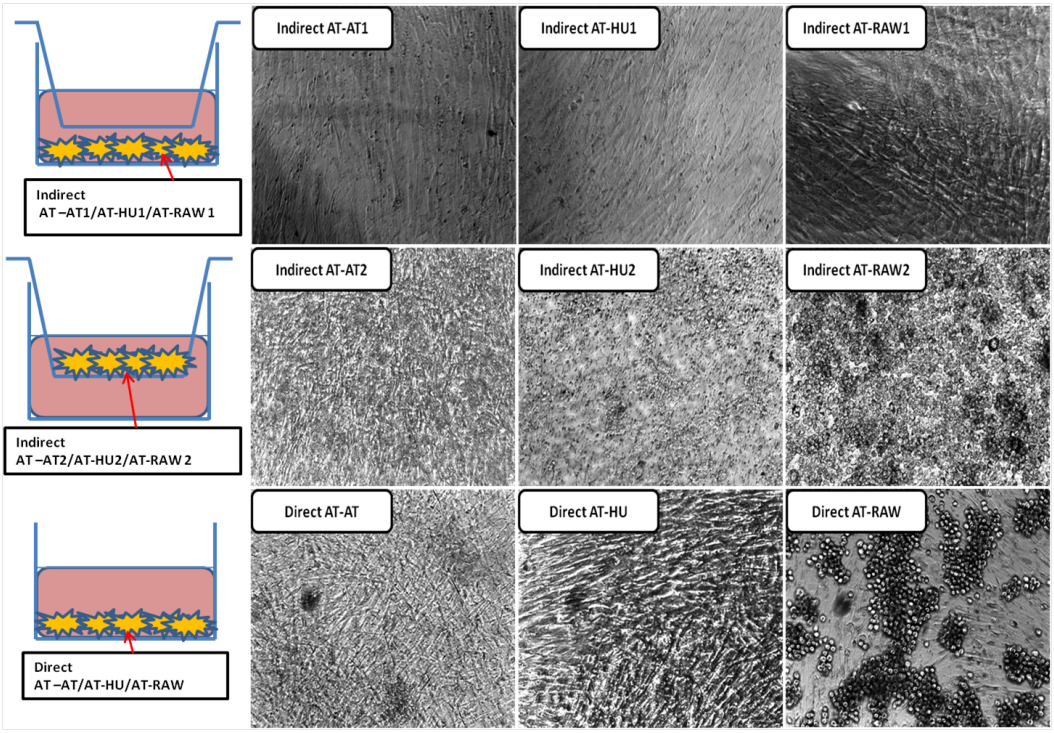


Figure 4.1: Cell morphology at day 7 (inverted light microscopy). The groups: AT-AT (control), AT-HU, AT-RAW in direct or indirect culture set-up

Already at day 7, cells completely covered the culture area of 24 well plates in both direct and indirect culture set-up. Detectable morphological differences were observed also in inserts (indirect culture set-up), especially for AT-MSCs versus RAW264.7 cells. AT-MSCs showed an elongated and spindle shape morphology, whereas RAW 264.7 cells appeared round shape. At day 35, dense cellular accumulations were observed for all groups, in which mineral depositions (black spots; highlighted with red arrows) were detectable (Figure 4.1 and 4.2).

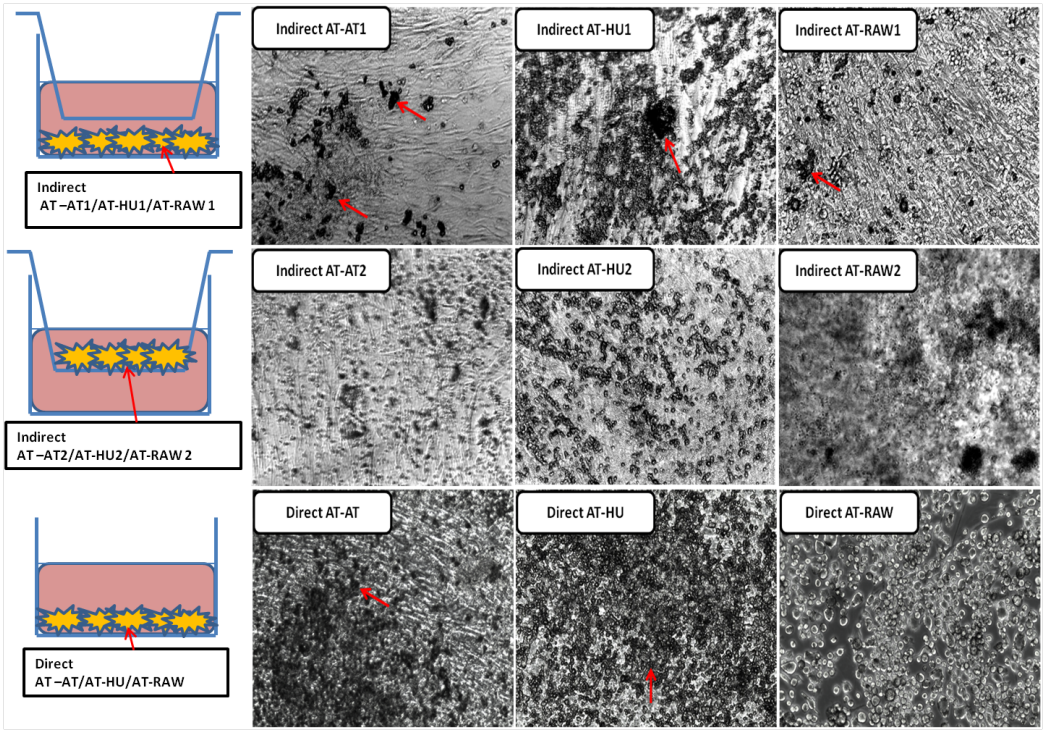


Figure 4.2: Cell morphology at day 35 (inverted light microscopy). The groups: AT-AT (control), AT-HU, AT-RAW in direct or indirect culture set-up.

Scanning electron microscopy showed that AT-MSCs (AT-AT) had a similar morphology in both direct and indirect co-culture where cellular multilayers covered globular deposits (Figure 4.3). In contrast, the direct and indirect co-cultures of AT-HU, as well as AT-RAW showed different morphologies. In direct AT-HU, many globular deposits were observed to which cells attached, but in indirect AT-HU only few globular deposits were detectable, which were covered by cellular multilayers. In direct AT-RAW, few cellular accumulations were observed and the culture surface appeared not completely covered with cells. In indirect AT-RAW, cells created very dense cellular multilayers with few globular deposits.

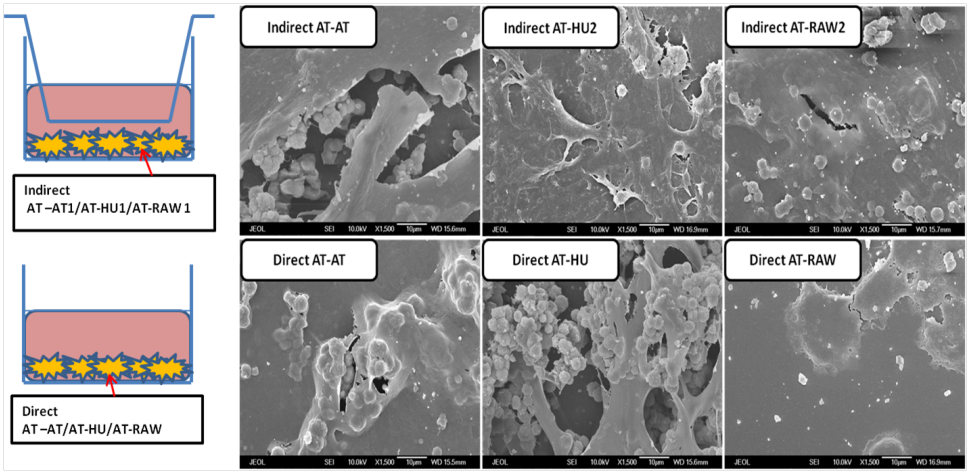


Figure 4.3: Cell morphology at day 35 (SEM). The groups: AT-AT (control), AT-HU, AT- RAW in direct or indirect culture set-up.

4.3.2 Effect of HU & RAW cells on AT-MSC proliferation

Based on results of cellular DNA content, a significantly increased proliferation was observed for direct AT-RAW ($p < 0.001$) during the entire culture period (Figure 4.4). The proliferation pattern for direct and indirect AT-HU, as well as for indirect AT-RAW was similar to AT-MSC monoculture, where proliferation increased till day 21 and day 28, after which proliferation decreased to day 35. Remarkably, in these groups (direct/indirect AT-AT, direct/indirect AT-HU, indirect AT-RAW) the proliferation was decreased also at day 14 (results of Donor 1). Table 4.3 presents an overview of co-culture results from 3 different donors, which shows similar proliferation patterns for all donors. Comparing direct versus indirect co-cultures (Table 4.4), an increased proliferation was generally observed for indirect AT-HU (Donor 2 and 3), and direct AT-RAW (all donors).

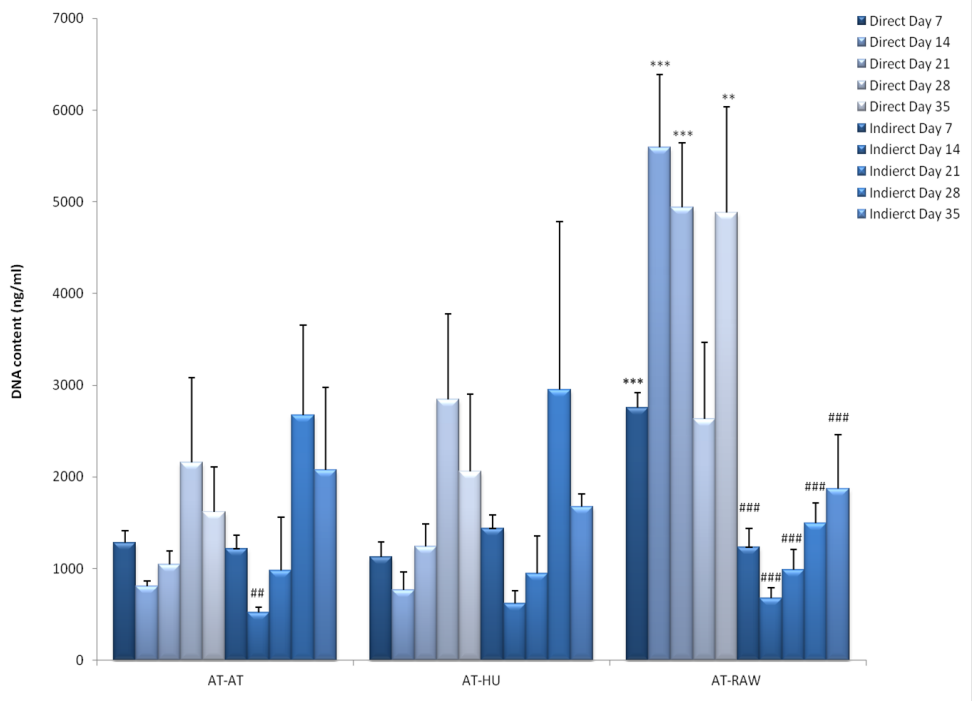


Figure 4.4: DNA content of AT-MSCs (Donor 1) at day 7, 14, 21, 28 and 35. The groups: AT-AT (control), AT-HU, AT-RAW in direct or indirect culture set-up. *p < 0.05, **p < 0.01 and ***p < 0.001 indicate the significant differences of AT-HU and AT-RAW compared to AT-AT control, in direct and indirect culture set-up. $^{\#}$p < 0.05, $^{\#\#}$ p< 0.01 and $^{\#\#\#}$p< 0.001 indicate the significant difference between direct and indirect co-culture.

4.3.3 Effect of HU & RAW cells on AT-MSCs differentiation

Early osteogenic differentiation of AT-MSCs was measured by ALP-activity for both direct and indirect culture set-up (Figure 4.5). The results indicated a positive effect of HUVECs on the osteogenic differentiation of AT-MSCs in both direct and indirect culture set-up, where the ALP level was significantly increased during the entire culture period (direct co-culture $\rightarrow$ p < 0.01 at day 7, 14, 28; p< 0.001 at day 21 and 35; indirect co-culture $\rightarrow$ p < 0.01 at

day 14; p< 0.05 at day 28). RAW264.7 cells also positively affected AT-MSCs osteogenic differentiation, but not during the entire culture period.

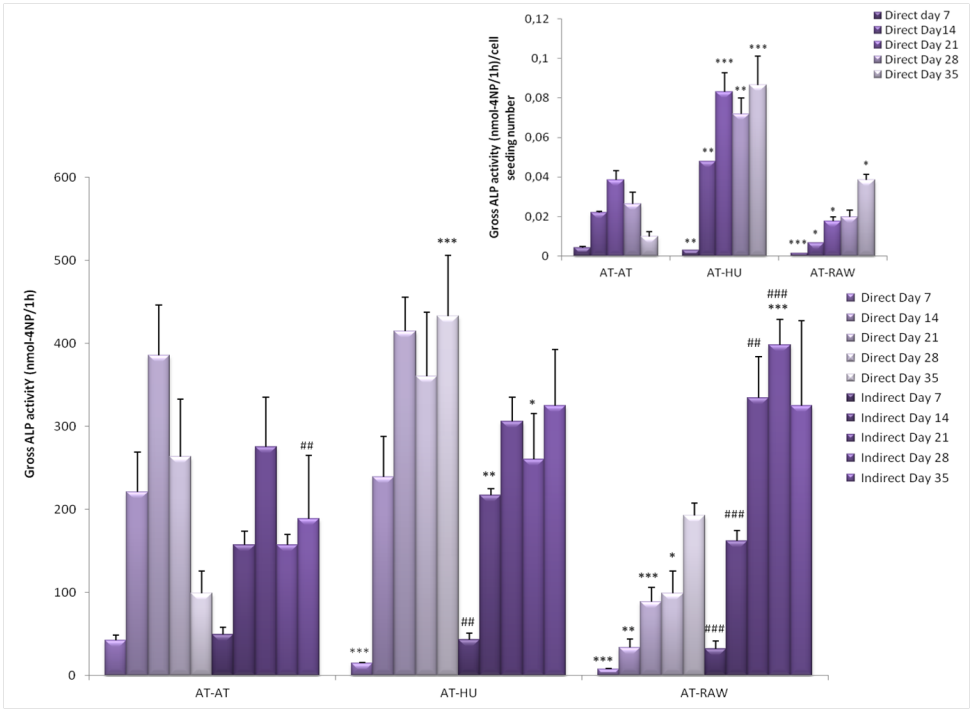


Figure 4.5: ALP activity of AT-MSCs (Donor 1) at day 7, 14, 21, 28 and 35. The groups: AT-AT (control), AT-HU, AT-RAW in direct or indirect culture set-up. *p < 0.05, **p < 0.01 and ***p < 0.001 indicate the significant differences of AT-HU and AT-RAW compared to AT-AT control, in direct and indirect culture set-up. #p < 0.05, ###p < 0.01 and ###p < 0.001 indicate the significant difference between direct and indirect co-culture.

A significantly increased ALP-activity was observed for direct AT-RAW at day 28 (p< 0.001) and for indirect AT-RAW at day 35 (p< 0.05). Next, a decreased osteogenic differentiation was observed during the first three weeks for direct AT-RAW (p< 0.001 → day 7; p< 0.05 → day 14 and 21). At the same time points, no significant differences were observed for indirect AT-RAW. ALP-activity was similar for the three different donors in direct AT-HU and direct AT-RAW, but varied for indirect AT-HU and indirect AT-RAW (Table 4.3). For direct versus indirect co-cultures, an increased

differentiation was observed for indirect AT-RAW (for all donors), and direct AT-HU (Donor 2, Donor 3).

4.3.4 Effect of HU & RAW cells on AT-MSCs mineralization

Ca deposition was measured to monitor the late stage osteogenic differentiation and mineralization of AT-MSCs.

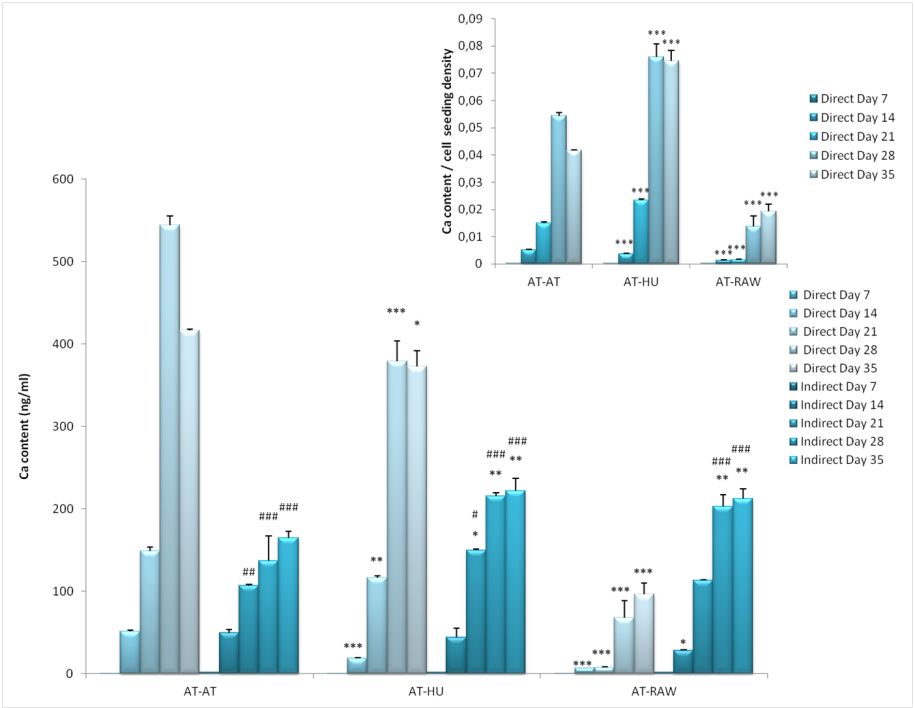


Figure 4.6: Ca content of AT-MSCs (Donor 1) at day 7, 14, 21, 28 and 35. The groups: AT-AT (control), AT-HU, AT-RAW in direct or indirect culture set-up. *p < 0.05, **p < 0.01 and ***p < 0.001 indicate the significant differences of AT-HU and AT-RAW compared to AT-AT control, in direct and indirect culture set-up. #p < 0.05, ##p < 0.01 and ###p < 0.001 indicate the significant difference between direct and indirect co-culture.

Results of Ca deposition were normalized for initial cell seeding number (Fig-

ure 4.6), which showed that HUVECs positively affected AT-MSCs mineralization in both direct and indirect culture set-up. For direct AT-HU, a significantly increased mineralization was observed at days 14, 21, 28 and 35 ($p< 0.001$). For indirect AT-HU, an increased mineralization was observed at days 21 ($p< 0.05$), 28 and 35 ($p< 0.01$). The mineralization patterns were similar for all three donors in the direct culture set-up, but varied in the indirect culture set-up (Table 4.3). Direct versus indirect co-cultures showed an increased mineralization for direct AT-HU for all three donors(Table 4.4). RAW 264.7 cells had a positive effect on AT-MSCs mineralization only in an indirect culture set-up (Donor1), in which a significantly increased mineralization was observed at days 14 ($p< 0.05$), 28 and 35 ($p< 0.01$). For direct AT-RAW, however, mineralization was significantly decreased at days 14, 21, 28 and day 35 ($p< 0.001$). The mineralization pattern of AT-RAW varied among the three donors (Table 4.3). Direct versus indirect co-culture showed an increased mineralization for all three donors (Table 4.4).

4.4 Discussion & Conclusion

The aim of this study was to evaluate the osteogenic differentiation of AT-MSCs upon co-culture with endothelial cells or macrophages in a direct or indirect culture set-up. Our hypotheses were that (i) endothelial cells and macrophages stimulate AT-MSCs proliferation and osteogenic differentiation, and that (ii) these two cell types will more profoundly affect on osteogenic differentiation of AT-MSCs in a direct compared to an indirect culture set-up, because of the possibility for both cell-cell interactions and effects of secreted soluble factors. We observed that HUVECs had a positive effect on osteogenic differentiation and mineralization of AT-MSCs, especially in a direct culture set-up, whereas the same HUVECs had no effect on AT-MSCs proliferation for neither direct nor indirect culture set-ups. Finally, we observed that RAW264.7 cells had a variable effect on AT-MSCs osteogenic

differentiation and mineralization.

An important concept in cell-based bone regenerative strategies is to understand cell-cell interactions between different cell types, which are involved in fracture healing, bone regeneration, remodeling and wound healing processes. This will help to investigate new approaches in bone regenerative research. To understand cellular communication between different cell types, this study used endothelial (HUVECs) and immune cells (RAW 264.7) as co-culture partners for AT-MSCs from three different donors. The results showed that HUVECs have a positive effect on AT-MSC osteogenic differentiation and mineralization, which was consistent for all three donors in direct co-culture set-up. A positive effect of AT-MSC osteogenic differentiation and mineralization was observed also for indirect AT-HU, but results were not consistent for all donors. This finding indicates that HUVECs can interact with AT-MSCs *in vitro* and promote osteogenic differentiation via direct and indirect cellular cross-talk [27, 28]. Moreover, in direct co-culture set-up, HUVECs more efficiently affected AT-MSCs osteogenic differentiation and mineralization compared to indirect set-up, likely because of simultaneous direct cell-cell contact and actions of secreted soluble molecules.

In contrast, HUVECs showed no effect on AT-MSC proliferation, neither in direct nor in indirect co-culture set-up. However, opposite results have been reported for MSCs isolated from bone tissue, for which HUVECs were able to increase the proliferation, especially in direct co-culture set-up [29]. These results suggest that MSCs from different tissue origins in co-culture with HUVECs may exert different behavior.

RAW264.7 cells showed a negative effect on AT-MSCs osteogenic differentiation and mineralization, especially in a direct co-culture set-up. Based on morphological evaluation of the co-culture, the number of AT-MSCs appeared very low for direct AT-RAW during the entire culture period. This could explain the low levels of ALP activity and Ca deposition in the direct

AT-RAW group. It is known that RAW264.7 cells are a type of macrophages, whose main function is to digest any substance recognized as foreign (cellular debris, pathogenic factors such as pathogen bacteria, viruses, cancer cells, etc.) in blood or in tissues [30]. Because AT-MSCs and RAW264.7 cells derive from different species, AT-MSCs might have been recognized as a foreign cell type and digested by RAW264.7 cells [31], which would explain survival issues of AT-MSCs in direct co-culture with RAW 264.7 cells. RAW264.7 cells further are able to differentiate into osteoclasts *in vitro* upon stimulation with RANKL. Osteoclasts are bone resorbing cells and generally in balance with osteoblasts. In direct contact with AT-MSCs, RAW264.7 cells might show osteoclastic behavior and affect AT-MSCs proliferation and differentiation into osteoblasts [32]. Although in the present study no proof was obtained for osteoclastogenesis of RAW264.7 cells, the occurrence of osteoclastic differentiation cannot be excluded, particularly in view of the used human platelet lysate during cell cultures, which is rich in many signaling molecules and growth factors.

For some AT-MSC donors, RAW264.7 cells showed a positive effect on osteogenic differentiation and mineralization in indirect co-culture set-up. Previously, a positive effect of macrophages on osteogenic differentiation of MSCs was shown by several studies. Few studies indicated that resident tissue macrophages (OsteoMacs) impact bone formation [33, 34]. Therefore, it becomes more interesting to investigate how macrophages (from the same species) can affect osteogenic differentiation of AT-MSCs *in vitro*. From all experimental groups, only direct AT-RAW showed an increased proliferation. Based on morphological appearance, however, this was likely a result of the fast growth of RAW264.7 cells rather than a stimulatory effect of macrophages on AT-MSCs proliferation.

The results of this study contribute to continuing and improving future in cell-based bone regenerative strategies, especially related to novel co-culture based strategies. The clinical relevance of this study is that AT-MSCs show

high potential for bone tissue regeneration, because AT-MSCs even at half the number of control mono-cultures, in co-culture with other cell types can achieve equal levels of *in vitro* mineralization compared to AT-MSC mono-cultures. Stimulated osteogenic differentiation and mineralization of AT-MSCs was observed especially for co-cultures with endothelial cells, where AT-HU 50%-50% ratio showed equal mineralization as AT-MSC monoculture. The fact that endothelial cells promote AT-MSCs differentiation into osteoblasts makes co-culture system attractive for future preclinical and clinical trials. This effective cellular communication gives an idea about cellular behavior in natural bone micro-environment. However, further research needs to be performed in co-culture systems and a variation of parameters must be investigated to mimic the natural bone micro-environment and entire regeneration cascade. By the help of dedicated scaffold systems, a 3D cellular interaction needs to be analyzed and more cell types need to be included in the same scaffold system. With successful *in vitro* results next, in vivo research need to be performed, where more parameters can be examined in a natural micro-environment.

In conclusion, endothelial cells showed a positive effect on AT-MSC osteogenic differentiation and mineralization. Interestingly, AT-MSCs in AT-HU co-culture set-up, where AT-MSCs were only 50% of total amount, compared to monoculture controls (100% AT-MSCs), achieved equal levels of mineralization. Direct co-culture of AT-MSCs with HUVECs appeared more effective for osteogenic differentiation and mineralization compared to indirect. This proved our hypothesis that direct co-cultures more profoundly affect osteogenic differentiation and mineralization of AT-MSCs because of direct cell-cell contact. Macrophages, for indirect co-cultures, affect on osteogenic differentiation and mineralization of AT-MSCs variably, whereas for direct co-cultures they decrease the osteogenic differentiation of AT-MSCs, which probably related to species differences in the used cell types. The results of this study demonstrate the potential of cell combination strategies in bone regenerative research.

4.5 Supplementary materials

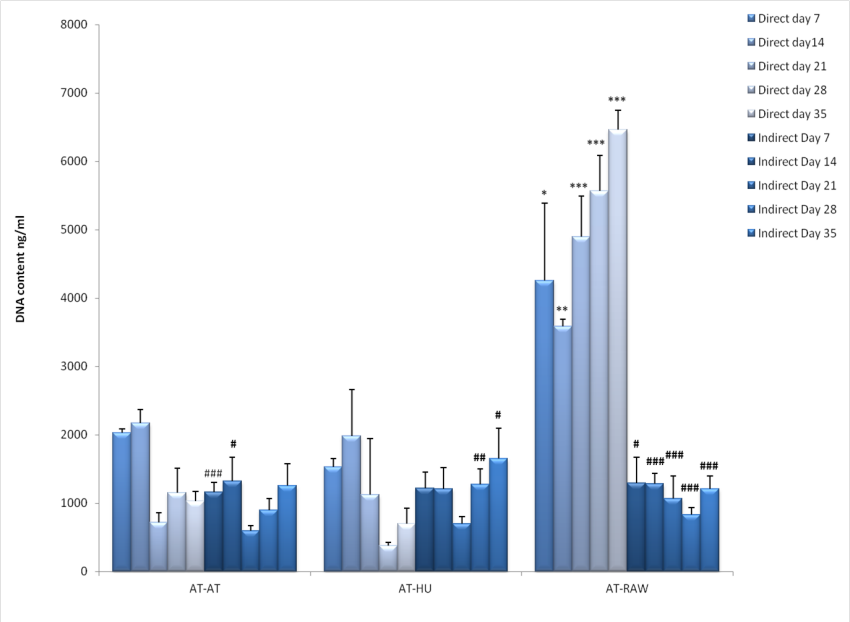


Figure 4.7: DNA content of AT-MSCs (Donor 2) at day 7, 14, 21, 28 and 35.

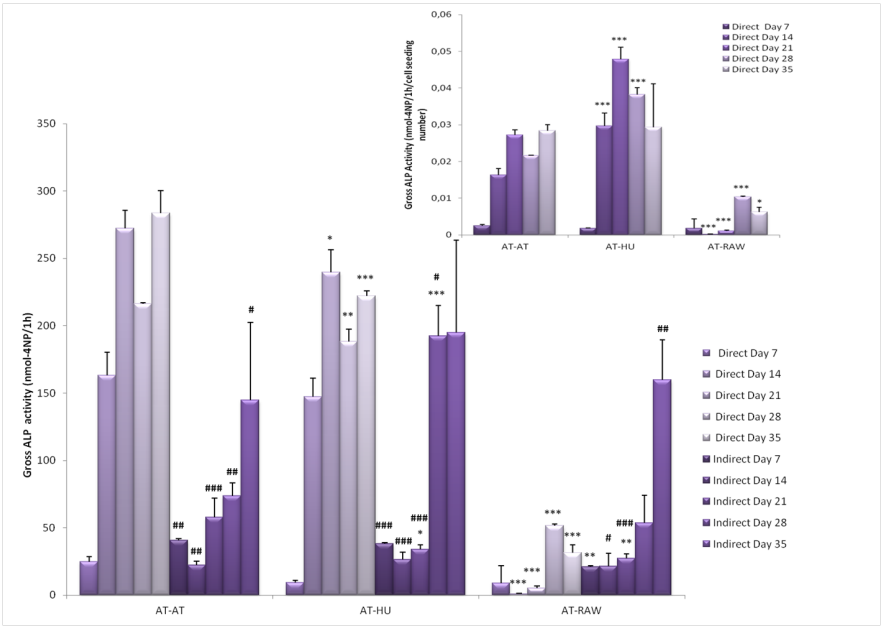


Figure 4.8: ALP activity of AT-MSCs (Donor 2) at day 7, 14, 21, 28 and 35.

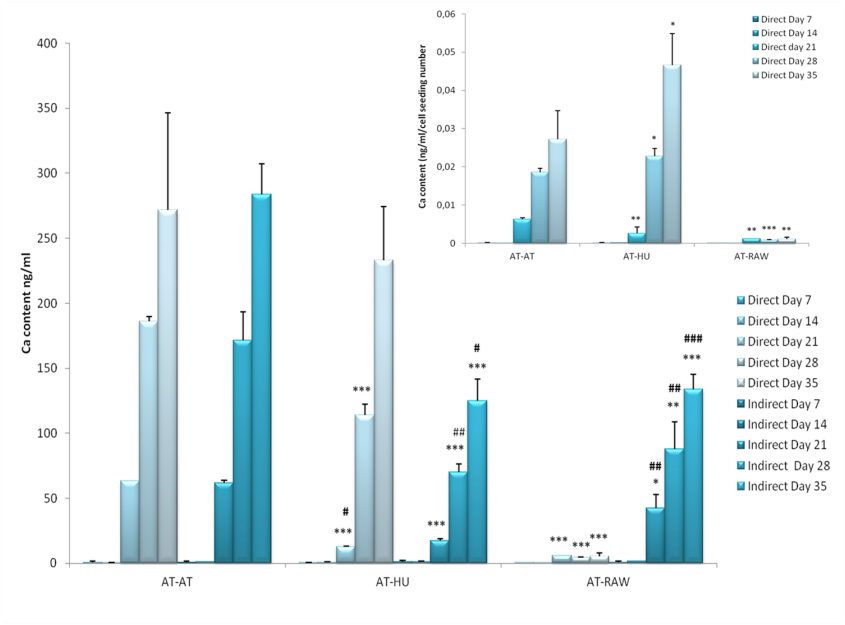


Figure 4.9: Ca content of AT-MSCs (Donor 2) at day 7, 14, 21, 28 and 35.

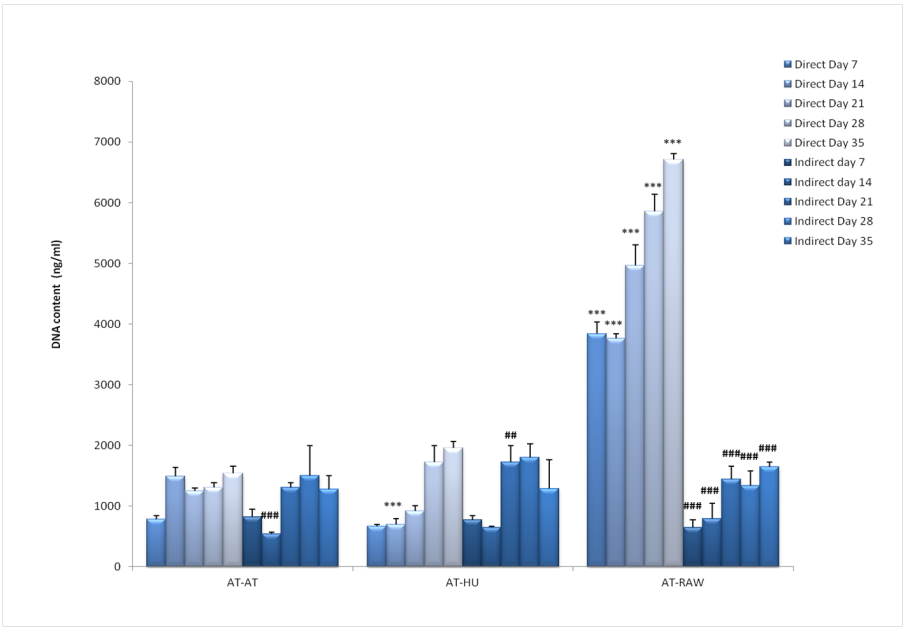


Figure 4.10: DNA content of AT-MSCs (Donor 3) at day 7, 14, 21, 28 and 35.

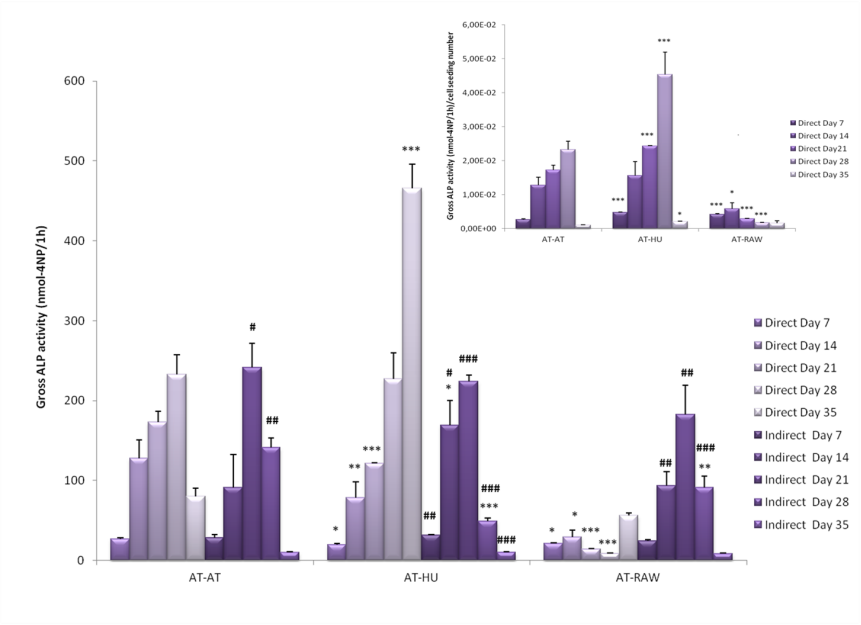


Figure 4.11: ALP activity of AT-MSCs (Donor 3) at day 7, 14, 21, 28 and 35.

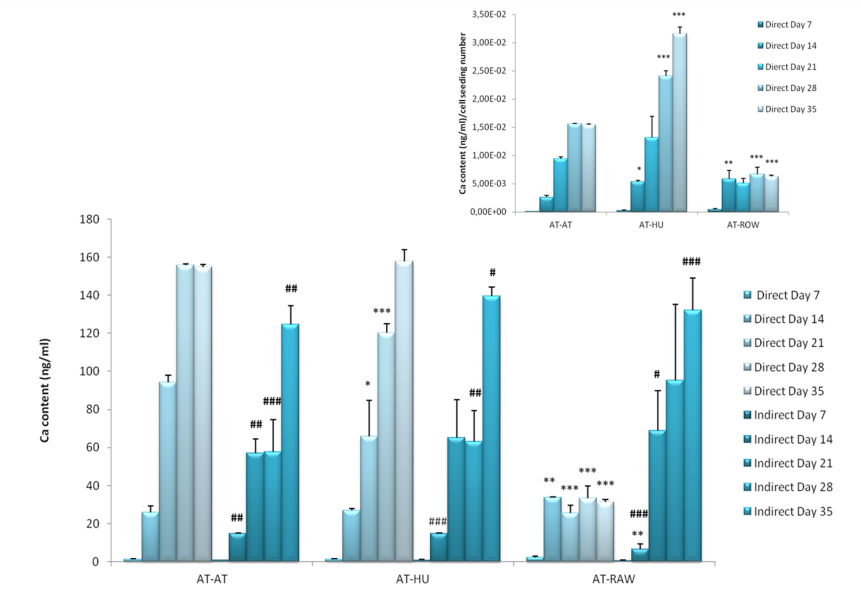


Figure 4.12: Ca content of AT-MSCs (Donor 3) at day 7, 14, 21, 28 and 35.

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CHAPTER 5

Effect of nano-HA/Collagen Composite Hydrogels on Osteogenic Behavior of Mesenchymal Stromal Cells

5.1 Introduction

Bone loss, caused by trauma, tumor resection or congenital disorders, is an increasingly serious health problem, for which current treatment remains a clinical challenge, especially for critical size bone defects [1]. Structurally, bone tissue forms the human skeleton, which consists of multiple cell types and a largely mineralized extracellular matrix (ECM). The organic part of ECM is composed of protein fibers (mainly type 1 collagen), which represents about 30 wt% of bone. The inorganic part of bone is composed of minerals (∼70 wt% of bone), with hydroxyapatite (HA; $Ca_{10}(PO_4)_6(OH)_2$) as the major component [2, 3]. As such, the ratio between collagen and HA is approximately 1:2. These HA crystals, embedded within the extracellular matrix, are very small, measuring approximately 200Å (in the largest dimension, i.e. nano-sized HA, nHA). In a synergistic manner, the strands of collagen provide bone with tensile strength and the interspersed nHA crystals

provide compressional strength [4].

Nowadays in bone regenerative research, the use of scaffold systems receives remarkable attention, because of the increasing demand to replace autologous bone for grafting purposes. For an appropriate cell attachment, proliferation and osteogenic differentiation, scaffolds need to pass several properties, e.g. biocompatibility, ability of fluid transport, delivery of bioactive molecules, surface topographical cues, degradability and ability to induce signal transduction [5].

In view of biomimicry, it seems appealing to develop scaffolds that combine structural properties of bone ECM. Among the different materials available for scaffold preparation, hydrogels represent a highly versatile group of biomaterials with appealing properties for 3D scaffolding. Hydrogels are hydrophilic networks (water content $\geq 30\%$ by weight) of natural or synthetic polymer chains, which approximate the viscoelastic properties of native tissue [6]. Because of distinctive features, such as biocompatibility, cell-controlled degradability, injectability, ability to release drugs or bioactive molecules, hydrogels are considered as reliable biomaterials for the regeneration of a wide range of tissues, including cartilage and bone [5, 7]. Natural hydrogels (i.e. collagen, fibrin) have more advantages, because they demonstrate non antigenic and intrinsic cellular interaction capacities [8]. Moreover, collagen based hydrogels support the expression of an osteogenic phenotype of (differentiating) MSCs *in vivo* [9]. However, the mechanical properties of collagen are relatively low (E$\sim$100MPa) in comparison to bone ($E \sim 2-50$GPa) [10]. To obtain more biomimetic scaffold systems for bone regeneration, several attempts have focused on the combination of a hydrogel and HA to study effects of cell behavior (mainly mesenchymal stromal cells, MSCs) *in vitro* and bone regeneration *in vivo* [11, 12, 13]. These studies have shown that HA/collagen based composite hydrogels can have potential to enhance MSC osteogenic differentiation [7, 14].

From a cellular perspective, hydrogels provide a 3D micro-environment to which cells can attach, attain a specific morphology, have 3D cell-cell/cell-biomaterial interactions and subsequently proliferate and differentiate [15]. Moreover, for bone regeneration, the use of cell-based constructs provides osteoinductive properties [16, 17] in comparison to bare scaffolds [18, 19].

Since in the developmental and regenerative processes of bone BM-MSCs are involved, they have become the main cell source for bone tissue engineering [20]. However, MSCs can be extracted from different tissues, such as skin, muscle, periodontal ligament, blood, adipose tissue (AT) and the yield of extracted cells is dependent of cell source. The easiest harvesting of MSCs (less invasive) with substantial yield is from adipose tissue [21, 22]. Moreover, at the same 2D culture condition (using platelet lysate [PL] or fetal bovine serum [FBS] as nutritional supplement), AT-MSCs showed higher proliferation and osteogenic differentiation capacities compared to BM-MSCs, and AT-MCSs showed their highest proliferation and osteogenic differentiation capacities in PL supplemented media, whereas BM-MSCs did in FBS supplemented media [21, 23]. In view of this, the major challenge remains to understand the complexity of cellular responses of different MSCs to different scaffold systems.

The aim of this study was to comparatively evaluate the *in vitro* effect of biomimetic nHA/collagen based composite hydrogels (with different ratios of nHA) on the behavior of human MSCs, isolated from adipose tissue (AT-MSCs) or bone marrow (BM-MSCs). We hypothesized that (i) nHA/collagen based hydrogels will promote the osteogenic differentiation of MSCs in an nHA concentration dependent manner, (ii) AT-MSCs will show higher osteogenic potential compared to BM-MSCs, because of their intrinsic higher proliferation and osteogenic differentiation potential in *in vitro* 2D cultures.

5.2 Materials & Methods

5.2.1 Cell culture

AT-MSCs were isolated from fat tissue of healthy human donors. The fat tissue was obtained from the Department of Plastic Surgery (Radboudumc, the Netherlands) after written informed consent.

Table 5.1: Composition of the proliferation media (PM) and osteogenic media (OM).

BM-MSCs	AT-MSCs
FBS-supplemented (PM-FBS)	PL-Supplemented (PM-PL)
Minimal Essential Medium (α-MEM) 15% fetal bovin serum (FBS) 0.2 mM L-ascorbic acide 2-phosphate (Vit C) 2 mM L-glutamine 100 U/ml penicillin 10 μg/ ml streptomycin	Minimal Essential Medium (α-MEM) 5% platelet lysate (PL) 10U/ml heparin 100 U/ml penicillin 10 μg/ ml streptomycin
FBS-supplemented (OM-FBS)	PL-Supplemented (OM-PL)
Minimal Essential Medium (α-MEM) 15% fetal bovin serum (FBS) 0.2mM L-ascorbic acide 2-phosphate (Vit C) 2 mM L-glutamine 100 U/ml penicillin 10 μg/ ml streptomycin 10-8 M dexamethasone 0.01 M β-glycerophosphate	Minimal Essential Medium (α-MEM) 5% platelet lysate (PL) 0.2 mM L-ascorbic acide 2-phosphate (Vit C) 2 mM L-glutamine 100 U/ml penicillin 10 μg/ ml streptomycin 10-8 M dexamethasone 0.01 M β-glycerophosphate 0.02 10U/ml heparin

BM-MSCs were isolated from human iliac bone chips, obtained from patients undergoing maxillofacial surgery at the Department of Oral and Craniofa-

cial Surgery (Radboudumc, the Netherlands) after written informed consent. MSC extraction was performed according to the principles of the Declaration of Helsinki. The isolation procedure of AT-MSCs and BM-MSCs is described in detail elsewhere [21, 24, 25, 26]. Harvested cells were examined by fluorescence-activated cell sorting (FACS) for positive expression of CD73, CD90 and CD105 (eBioscience, San Diego, USA) and negative expression of CD45 (R&D system, Abingdon, United Kingdom). Next, both cell types were examined (via biochemical assays) for their osteogenic potential, i.e. ALP activity and calcium deposition. Upon usage, MSCs were cultured in corresponding proliferation media consisting of alpha Minimal Essential Medium, (α-MEM; Gibco®, Life Technologies, Grand Island, USA) supplemented either with 5% PL (Sanquin Blood Bank, the Netherlands) for AT-MSCs or with 15% fetal bovine serum (FBS; Lonza, Basel, Switzerland) for BM-MSCs [21], at 37°C in humid atmosphere with 5% CO2 (the complete composition of proliferation media is given in Table 5.1). Medium was changed twice a week. Cells were passaged upon reaching $\sim$ 80% confluency using 0.25% w/v trypsin/0.02% EDTA (Gibco®).

5.2.2 Preparation of hydrogels & experimental groups

Prior to the preparation of hydrogel scaffolds, nHA crystals (size: 20-500 nm; Berkeley Advanced Biomaterials, Berkeley, CA, USA) were suspended in PBS (10x concentrated) with a final concentration of 150 mg/ml. The suspension was homogenized by sonication for 20 min. Before addition to hydrogels (see in Table 5.2), each time the suspension was vortexed for 1 min. For the preparation of hydrogels, collagen type 1 (COL; rat tail; BD Bioscience, Bedford MA, USA) was used with various amounts of nHA (Table 5.2). The procedure of hydrogel preparation was according to manufacturer's instruction (Table 5.2), and composite nHA/COL hydrogels were prepared with an nHA/COL ratio (wt/wt) of 0/1, 1/1, and 2/1. MSCs were added during hydrogel preparation (Table 5.2).

Table 5.2: Reagents for scaffold preparation and cell encapsulation.

Groups	A. Without cells	B. With the cells
CaP/ Collagen 0:1 (control)	Collagen 2610 μl PBS(10x) 300μl CaP susp. 0 NaOH 1N 60μl H2O/α-MEM 30μl Cell susp. 0 Total 3000 μl	Collagen 2610 μl PBS(10x) 300μl CaP susp. 0 NaOH 1N 60μl H2O/α-MEM 0μl Cell susp. 30μl Total 3000 μl
CaP/ Collagen 1:1	Collagen 2610 μl PBS(10x) 240μl CaP susp. 60μl (150 mg/ml) NaOH 1N 60μl H2O/α-MEM 30μl Cell susp. 0 Total 3000 μl	Collagen 2610 μl PBS(10x) 240μl CaP susp. 60μl (150 mg/ml) NaOH 1N 60μl H2O/α-MEM 0μl Cell susp. 30μl Total 3000 μl
CaP/ Collagen 2:1	Collagen 2610 μl PBS(10x) 180μl CaP susp. 120μl (150 mg/ml) NaOH 1N 60μl H2O/α-MEM 30μl Cell susp. 0 Total 3000 μl	Collagen 2610 μl PBS(10x) 180μl CaP susp. 120μl (150 mg/ml) NaOH 1N 60μl H2O/α-MEM 0μl Cell susp. 30μl Total 3000 μl

Cell seeding density of AT-MSCs and BM-MSCs in all experimental groups was 1x10^6 per 1 ml of hydrogels. For the analysis of cellular behavior (DNA content, ALP activity and calcium [Ca] deposition) and histological analysis (HE staining, Von Kossa staining and immunohistochemistry [IHC]) hydrogels were injected in 48 well plates, with the total hydrogel volume of 200 μl (200.000 cells; n=3). To obtain sufficient RNA, hydrogels for RNA extraction were injected in 24 well plates, with the total volume of 400 μl (400.000

cells; n=3). All samples were incubated in corresponding osteogenic media (Table 5.1), supplemented with either 5% PL for AT-MSCs or 15% FBS for BM-MSCs and incubated for 35 days at 37°C in a humid atmosphere with 5% CO2. To monitor the behavior of pure hydrogels (without cells) as a negative control nHA/COL=0/1, nHA/COL=1/1, nHA/COL=2/1 constructs were prepared and cultured either in PL or in FBS supplemented media. Cell morphology was monitored with an inverted light microscope (Leica DM-IL, 5W LED illumination, Rijswijk, the Netherlands).

5.2.3 Cell behavior

To monitor cellular behavior, cellular DNA content, alkaline phosphatase (ALP) activity and calcium deposition were analyzed [21]. Samples were collected (at days 1, 14, 28 and 35) in 1 ml MilliQ and subsequently stored at −80°C until use. The same samples were used for all biochemical assays. For extraction of cells from hydrogels, scaffolds were digested first using Collagenase A (Roche Diagnostics Penzberg, Germany). According to the manufacturer's instruction, a digestion buffer was prepared in a concentration of 1 mg/ml. Before digestion, the constructs were washed 2 times with PBS, incubated with 1 ml digestion buffer overnight at 37°C on a rotary shaker. After complete digestion and two repetitive freeze/defroze cycles at −80°C/ + 20°C, samples were ready for the analysis of DNA content, ALP activity and Ca deposition [21, 27]. After analyzing DNA content and ALP activity, samples were doubled in volume using 1N acetic acid (to dissolve mineral deposits) and incubated at room temperature overnight. Ca deposition was measured as described previously [28].

5.2.3.1 Cellular DNA content

Cellular DNA content was measured using a QuantiFluor® dsDNA System Kit (Promega, Madison, Wisconsin, USA). For the standard curve, serial dilutions of dsDNA stock (range: 0-2000 ng/ml) were prepared. 100 μl of

either sample of standard solution was added into the wells, followed by 100 μl of working solution. The plate was incubated at room temperature for 5 min, and then the absorbance of samples/standards was measured at 504 nm excitation and 541nm emission, using a fluorescence microplate reader (FL600, BioTek, Canada).

5.2.3.2 Alkaline phosphatase (ALP) activity

ALP activity was measured using a 5 nM p-nitrophenyl phosphate (4-NP) colorimetric assay. The procedure was according to the manufacturer's instruction (Sigma-Aldrich, St. Louis, MO, USA). Briefly, 80 μl of sample solution was combined with 20 μl of buffer (0.5 M 2-amino-2methyl-1-propanol). A standard curve was prepared with serial dilutions of 4-NP, in a range of 0-25 nmol. Next, 100 μl substrate solution (5 nM p-nitrophenyl phosphate) was added to the samples/standards and incubated for 60 min at 37°C. The reaction was stopped by adding to each well 50 μl 0.3M NaOH and the absorbance of samples was measured at 405 nm using an ELISA microplate reader (EL800, BioTek, Abcoude, the Netherlands). ALP activity was normalized for corresponding cellular dsDNA amount.

5.2.3.3 Calcium deposition

Calcium deposition was measured using the orthocresolphtalein complexone assay (OCPC; Sigma Aldrich, St. Louis, MO, USA), which is based on a colorimetric reaction between o-cresolphthalein complexone and calcium. The assay was performed according to the manufacturer's instruction. For the standard curve, serial dilutions of calcium stock (CaCl2) were prepared (range: 0-100 mg/ml). Next, 10 μl of sample or standard was used, to which 300 μl OCPC solution was added to complete the reaction. After the incubation of the plate for 10 min at room temperature, the absorbance

was measured at 570 nm using an ELISA microplate reader (EL800, BioTek, Canada).

5.2.4 Analysis of osteogenic molecules

5.2.4.1 RNA isolation and reverse transcription

To analyze gene expression profiles of selected genes, cellular RNA was isolated using Tryzol method in combination with the Genelute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich). Samples were collected (at days 0, 14, 28 and 35) in 1 ml trizol lysis buffer and subsequently stored at $-80°C$ until RNA extraction. After extraction, RNA was quantified using a spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). For reverse transcription (cDNA synthesis), an iScript^{TM} cDNA kit was used (Bio-Rad, Hercules, CA, USA), and for each cDNA reaction 1 μg of RNA was used. The samples were stored at $-20°C$ until use.

5.2.4.2 Real-Time Polymerase Chain Reaction

For real-time polymerase chain reaction (RT-PCR), qPCR Master Mix Plus/ SYBR Green I (Eurogentec; Seraing, Belgium) was used. RT-PCR was completed with 40 amplification cycles. The sequence of applied primers is given in Table 5.3. The raw data were normalized to the expression of Ribosomal Protein Large P0 (RPLP0 housekeeping gene) within the same sample/RNA [29]. Gene expression level and fold changes were calculated according to Livak & Schmittgen ($2^{-\Delta\Delta Ct}$) relative subsequently to AT-MSCs or BM-MSCs at the day 0 [30]. The genes of interest were Osteocalcin (OCN), Bone Morphogenetic Protein 2 (BMP2), Runt-related Transcription Factor 2 (RUNX2), and Collagen type 1 (COL1).

Table 5.3: Primer sequences for RT-PCR

Gene name	Sequences
RPLP0	Forward- TTCTTCTTTGGGCTG GTCAT Reverse- TTGGGTAGCCAATCTGCAGA
RUNX2	Forward- TCTGGCCTTCCACTCTCAGT Reverse- GACTGGCGGGGTGTAAGTAA
BMP-2	Forward- CCCAGCGTGAAAAGAGAGAC Reverse- GGAAGCAGCAACGCTAGAAG
COL1	Forward- GGTGTAAGCGGTGGTGGTTAT Reverse- AGGTTCCCCGTTCTCACTTT
OCN	Forward- GGCGCTACCTGTATCAATGG Reverse- GTGGTCAGCCAACTCGTCA

5.2.5 Histological analysis & immunohistochemistry

Samples for histological analysis and IHC were collected at day 7 and 28, fixed at 10% formalin, decalcified in 4% EDTA for $\sim$ 2 weeks, and regularly checked with X-ray for the level of remaining mineral content in the constructs. After complete demineralization, samples were dehydrated in a graded series of ethanol (70-100%) and embedded in paraffin. Simultaneously, human bone chips (obtained from the Department of Maxillofacial surgery, Radboudumc, Nijmegen, the Netherlands; after written informed consent), were processed as a control for all stains. Sections with a thickness of 6 μm were prepared using a standard microtome (RM2165; Leica, Nussloch, Germany). To analyze cell distribution in hydrogels, every 10^{th} slide was stained with hematoxylin/eosin (HE). To identify phosphate groups in mineralized matrix, separate slides were prepared for Von Kossa staining, which were stained first with 5% silver nitrate ($AgNO_3$), washed with distilled water, dehydrated again, and fixed with 5% sodium thiosulfaat ($Na_2S_2O_3$). Next, continuous tissue sections were used to monitor osteogenic differen-

tiation of cells in hydrogels. As an osteogenic marker, osteocalcin (OCN, rabbit anti-mouse osteocalcin) protein was cheeked by IHC. Sections were deparaffinised, rehydrated and rinsed in PBS. Next, samples were fixed for 10 min in 10% hydrogen peroxide (stock)/methanol solution. Afterwards, samples were pre-incubated for 10 min with 10% normal donkey serum (NDS) and then incubated with the primary antibody (1:1600) overnight at 4°C. Subsequently, sections were washed 3x with PBS, and incubated with secondary antibody, anti mouse IgG (host donkey) conjugated with biotin (Jackson Immuno-Research, West Baltimore Pike, West Grove, PA, USA) for 60 min. After washing, the peroxidase conjugates were visualized with 3'3 diaminobenzidine (DAB) substrate (Envision kit; Dako Cytomation) for 10 min at room temperature, and nuclei were stained for 10 sec with hematoxilin.

5.2.6 Statistical analysis

Data are presented as mean ± standard deviation. Statistical analysis was performed based on N=3 (for all experimental groups) with Graphpad Prism® 5.03 software (Graphpad Software Inc, San Diego, CA, USA). Quantitative results were analyzed using a one-way ANOVA with a Posthoc Dunnett test (using nHA/COL=0/1 either day 7 [for biochemical assays] or day 14 [for Q-PCR analysis] as control). Differences were considered significant at $p < 0.05$. All experiments were repeated 4 times.

5.3 Results

The results of FACS analysis showed that both cell types (AT-MSCs and BM-MSCs) were 99% positive for expression of stem cell surface markers CD73, CD90 and CD105, whereas both these cell types were completely negative for the hematopoietic marker CD45 (data not shown).

5.3.1 Cell morphology

Light microscopy analysis showed that both AT-MSCs and BM-MSCs were able to survive in nHA/COL constructs (Figure 5.1).

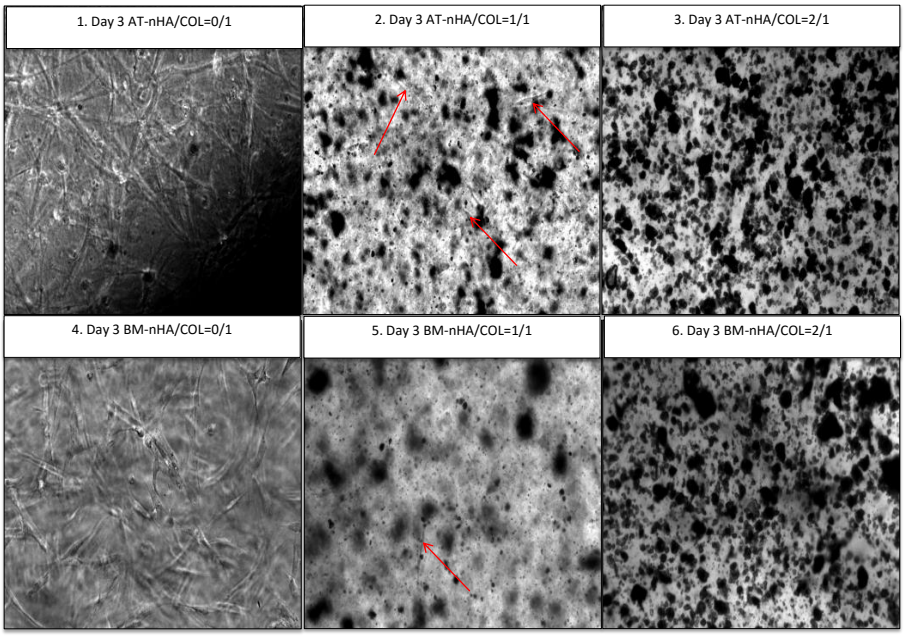

Figure 5.1: Cell morphology of AT-MSCs or BM-MSCs monitored with inverted light microscopy in different nHA/COL constructs, after 3 days of culture

Both cell types showed an elongated, spindle-shaped morphology in these constructs, but from 3 days of culture onward, AT-MSCs visually appeared at higher cell density than BM-MSCs. After 7 days, hydrogels changed their shape and morphology, becoming dense and no longer transparent for light microscopy evaluation. The constructs shrunk drastically by changing diameter size about 2-fold (from ∼11mm to ∼4-5mm).

5.3.2 Cell behavior

5.3.2.1 Cellular DNA content

AT-MSCs showed a gradual increase in cell proliferation until day 14 (Figure 5.2). A significant temporal increase in cellular DNA content (relative to day 7) was observed for AT-nHA/COL=0/1 at day 14 ($p<0.05$), AT-nHA/COL=1/1 at day 14, 28, 35 ($p< 0.001$), and AT-nHA/COL=2/1 at day 14, ($p< 0.001$). Relative to AT-nHA/COL=0/1, at day 7, a significantly lower cellular DNA content was observed for AT-nHA/COL=1/1 ($p< 0.05$) and AT-nHA/COL=2/1 ($p< 0.01$). BM-MSCs (Figure 5.2), showed limited cell proliferation over the entire culture period. Relative to day 7, a significant increase in cellular DNA content was observed only for BM-nHA/COL=2/1 at day 14 ($p< 0.05$).

5.3.2.2 ALP activity

AT-MSCs showed a gradual increase in ALP-activity (early marker for osteogenic differentiation) until the end of the culture period, irrespective of construct type (Figure 5.3). A significant increase in ALP-activity (relative to day 7) was observed for AT-nHA/COL=0/1 and AT-nHA/COL=1/1 at day 28 and 35 ($p< 0.001$), and AT-nHA/COL=2/1 at days 28 ($p< 0.01$) and 35 ($p< 0.001$). Relative to AT-nHA/COL=0/1, no significant differences were observed for AT-nHA/COL=1/1 and AT-nHA/COL=2/1. In contrast to AT-MSCs, very low ALP-activity was observed for BM-MSCs (Figure 5.3) over the entire culture period, which was significantly decreased (relative to day 7) until the end of culture in all experimental constructs. Relative to BM-nHA/COL=0/1, no significant differences were found between different constructs.

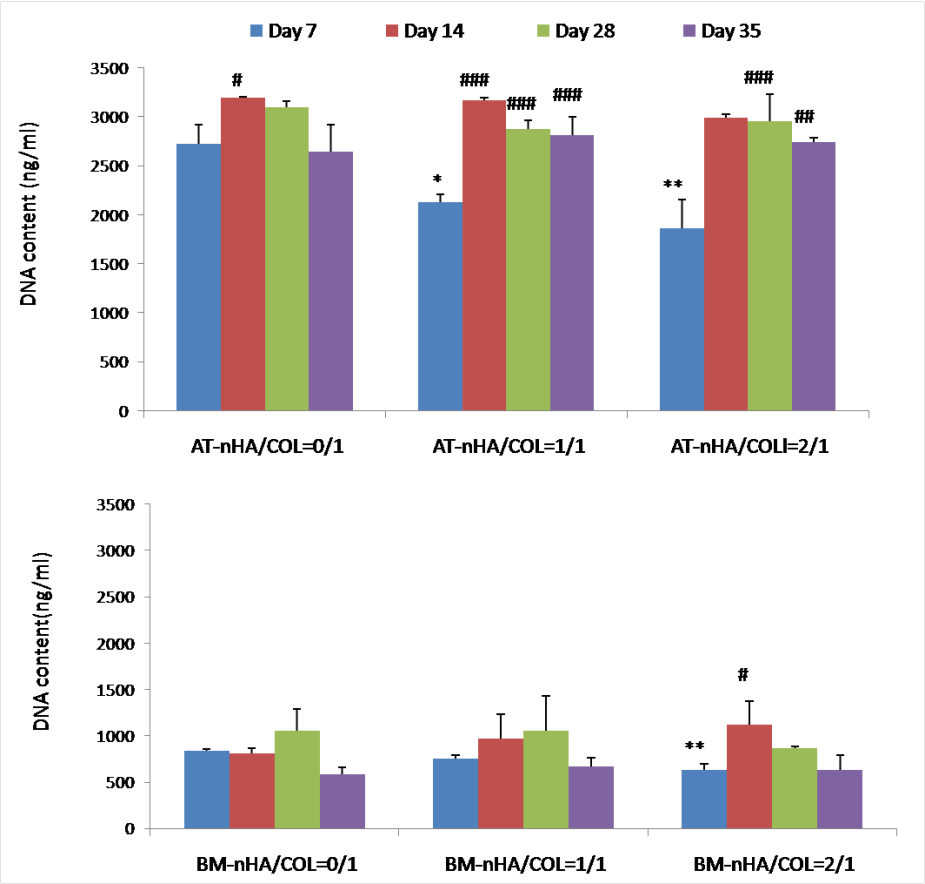

Figure 5.2: Cellular behavior of MSCs in different nHA/COL constructs. (A) Cellular DNA content of AT-MSCs, (B) Cellular DNA content BM-MSCs. The * indicates significantly different compared to nHA/COL=0/1 control, at the same time point (p-values: *p <0.05, ** p<0.01, *** p<0.001), # indicates significantly different compared to day 7 (p-values: #p <0.05, ## p<0.01, ### p<0.001).

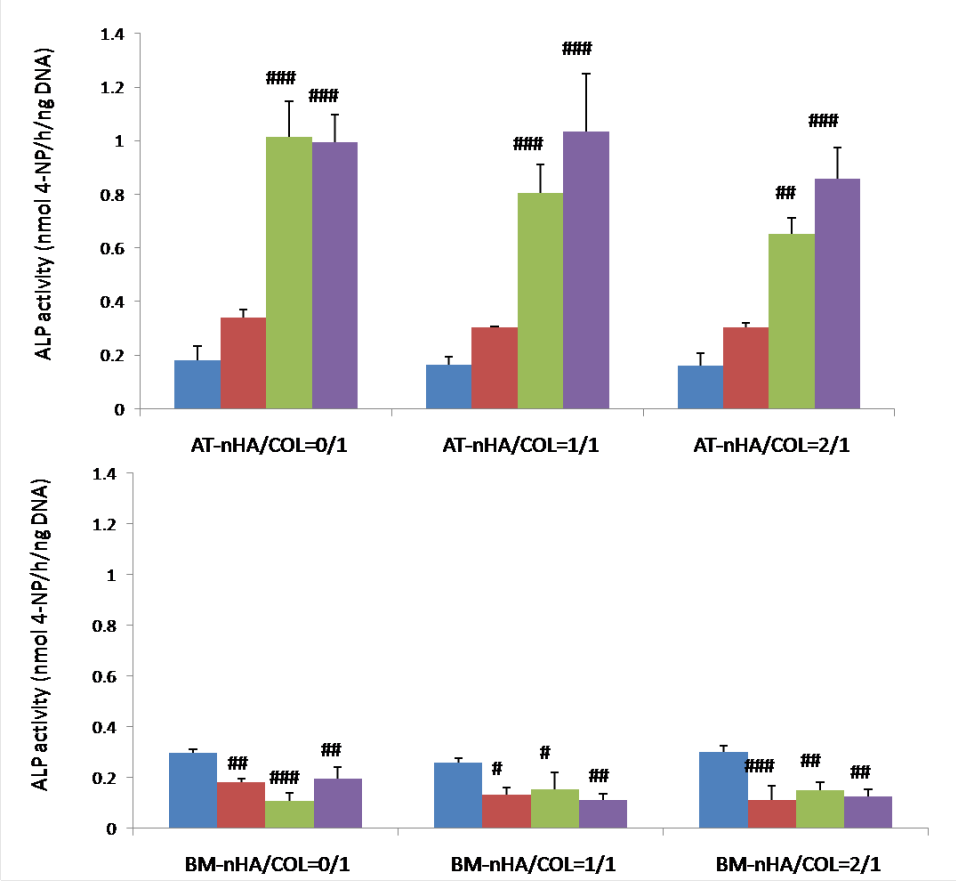

Figure 5.3: Cellular behavior of MSCs in different nHA/COL constructs. (A) ALP-activity of AT-MSCs,(B) ALP-activity of BM-MSCs. * indicates significantly different compared to nHA/COL=0/1 control, at the same time point (p-values: *p <0.05, ** p<0.01, *** p<0.001), # indicates significantly different compared to day 7 (p-values: #p <0.05, ## p<0.01, ### p<0.001).

5.3.2.3 Calcium deposition

Calcium deposition results (Figure 5.4 A,B) were normalized for non-cellular constructs, which were treated similarly as cellular constructs. The mineralization level for both cell types was generally low. Relative to day 7, AT-MSCs showed a temporal increase in calcium deposition for AT-nHA/COL=1/1

at day 28 ($p < 0.05$), and at day 35 ($p < 0.001$) for all experimental constructs.

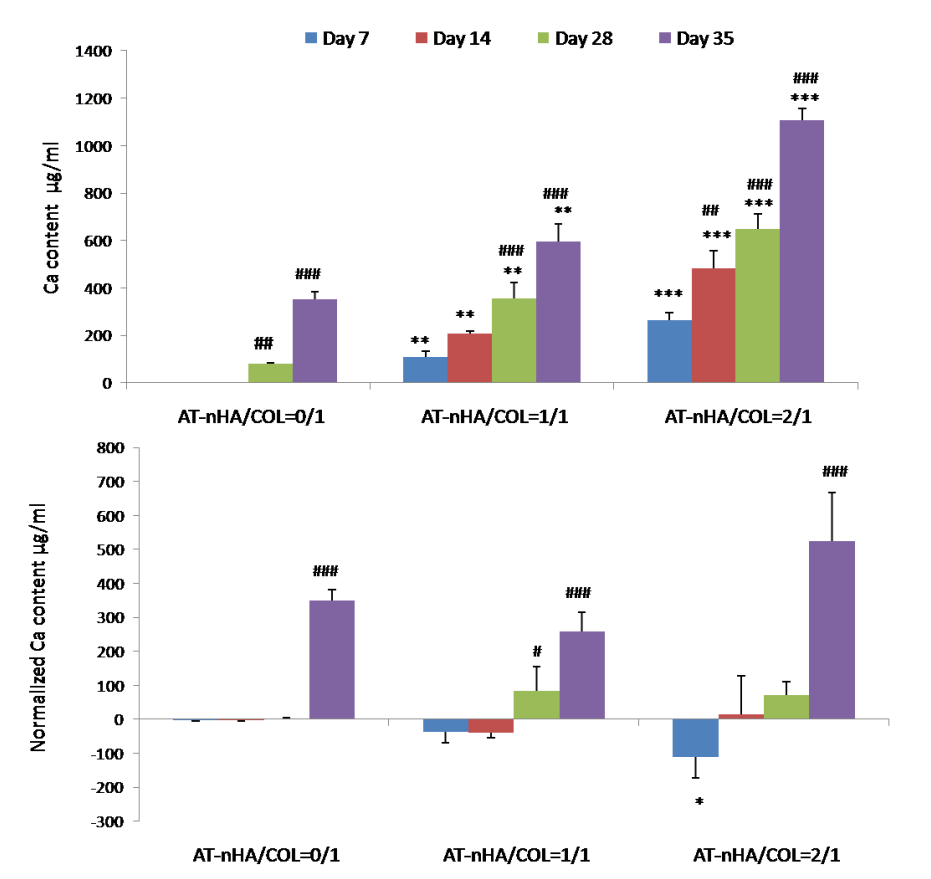

Figure 5.4: Ca deposition of MSCs in different nHA/COL constructs. (A) Ca deposition of AT-MSCs, (B) Normailzed Ca deposition of AT-MSCs. * indicates significantly different compared to nHA/COL=0/1 control, at the same time point (p-values: *p <0.05, ** p<0.01, *** p<0.001), # indicates significantly different compared to day 7 (p-values: #p <0.05, ## p<0.01, ### p<0.001).

BM-MSCs generally showed a decrease in mineralization (relative to day 7) starting at day 14 until the end of the culture period (Figure 5.5A,B). Only for BM-HA/COL=0/1, an increase in mineralization was observed at day 35 ($p < 0.001$). Relative to BM-nHA/COL=0/1, a significantly increased mineralization was observed for BM-nHA/COL=1/1 and BM-nHA/COL=2/1

($p< 0.001$) at day 7.

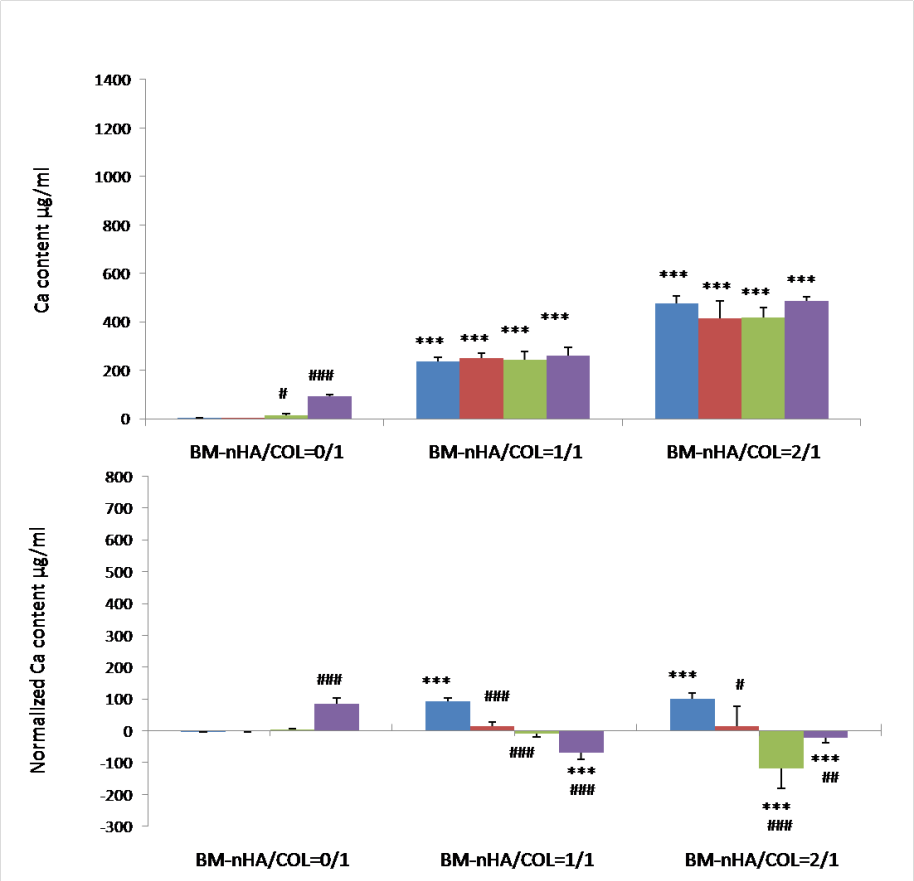

Figure 5.5: Ca deposition of MSCs in different nHA/COL constructs. (A) Ca deposition of BM-MSCs, (B) Normailzed Ca deposition of BM-MSCs. * indicates significantly different compared to nHA/COL=0/1 control, at the same time point (p-values: *p <0.05, ** p<0.01, *** p<0.001), # indicates significantly different compared to day 7 (p-values: #p <0.05, ## p<0.01, ### p<0.001).

5.3.3 Gene expression

BMP2: AT-MSCs showed a gradual increase in BMP2 expression until day 28, and decrease again until day 35, irrespective of construct type (Figure 5.6A).

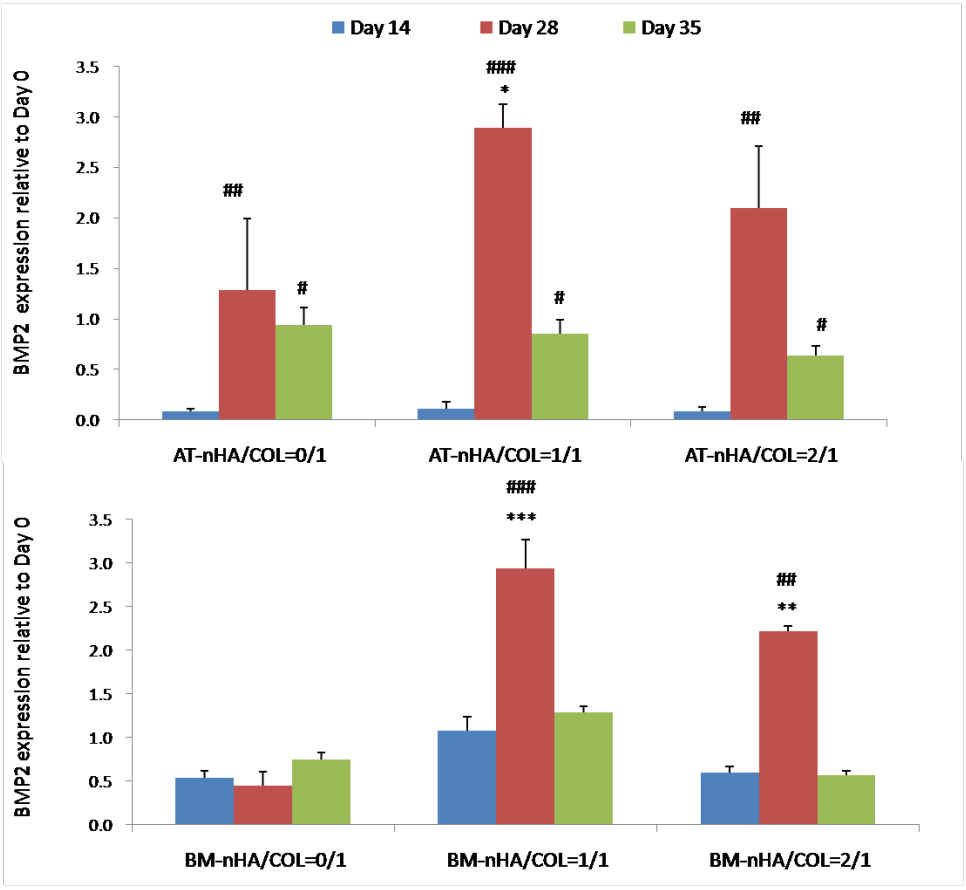

Figure 5.6: Gene expression profiles of BMP2 (A) in AT-MSCs and (B) in BM-MSCs. * indicates significantly different compared to nHA/COL=0/1 control, at the same time point (p-values: *p <0.05, ** p<0.01, *** p<0.001), # indicates significantly different compared to day 7 (p-values: #p <0.05, ## p<0.01, ### p<0.001)

Relative to day 14, significantly higher BMP2 expression was observed for AT-nHA/COL=0/1 at day 28 ($p< 0.01$) and 35 ($p< 0.05$), for AT-nHA/COL=1/1 at day 28 ($p< 0.001$) and 35 ($p< 0.05$), and for AT-nHA/COL=2/1 at day 28 ($p< 0.01$) and 35 ($p< 0.05$). Relative to AT-nHA/COL=0/1, significantly higher BMP2 expression was observed for AT-nHA/COL=1/1 at day 28 ($p< 0.05$). BM-MSCs showed a gradual increase in BMP2 expression until day 28 and thereafter a decrease until day 35 (Figure 5.6B) for BM-nHA/COL=1/1 and BM-nHA/COL=2/1. Relative to day 14, significantly

higher BMP2 expression was observed for BM-nHA/COL=1/1 and for BM-nHA/COL=2/1 at day 28 ($p< 0.001$). Relative to BM-nHA/COL=0/1, significantly higher BMP2 expression was observed for BM-nHA/COL=1/1 and for BM-nHA /COL=2/1 at day 28 ($p< 0.01$).

RUNX2: AT-MSCs showed an upregulation of RUNX2 expression after day 14, irrespective of construct type (Figure 5.7A). Relative to day 14, significantly higher RUNX2 expression was observed for AT-nHA/COL=1/1 at day 28 ($p< 0.05$) and for AT-nHA/COL=2/1 at day 28 ($p< 0.01$). BM-MSCs, showed high levels of RUNX2 expression at day 14 irrespective to construct type, which further increased only for BM-nHA/COL=1/1 (Figure 5.7B). Relative to day 14, significantly higher RUNX2 expression was observed for BM-nHA/COL=1/1 at day 28 ($p< 0.05$). Relative to BM-nHA/COL=0/1 significantly higher RUNX2 expression was observed for BM-nHA/COL=1/1 at day 28 ($p< 0.001$) and for BM-nHA/COL=2/1 at day 14 ($p< 0.001$).

OCN: AT-MSCs showed an upregulation of OCN expression starting at day 28 until day 35, except for AT-nHA/COL=2/1, for which the expression of OCN was downregulated after day 28 (Figure 5.8A). BM-MSCs, showed high expression of OCN at day 14, which was downregulated toward the end of the culture period, irrespective of construct type (Figure 5.8B). Relative to day 14, significantly lower OCN expression was observed for BM-nHA/COL=0/1 at day 35 ($p< 0.05$), for BM-nHA/COL=1/1 at day 28 ($p< 0.01$) and 35 ($p< 0.001$) and for BM-nHA/COL=2/1 at day 28 and 35 ($p< 0.05$). Relative to BM-nHA/COL=0/1, an upregulation of OCN expression was observed for BM-nHA/COL=1/1 at day 14 ($p < 0.05$) and 28 ($p< 0.01$).

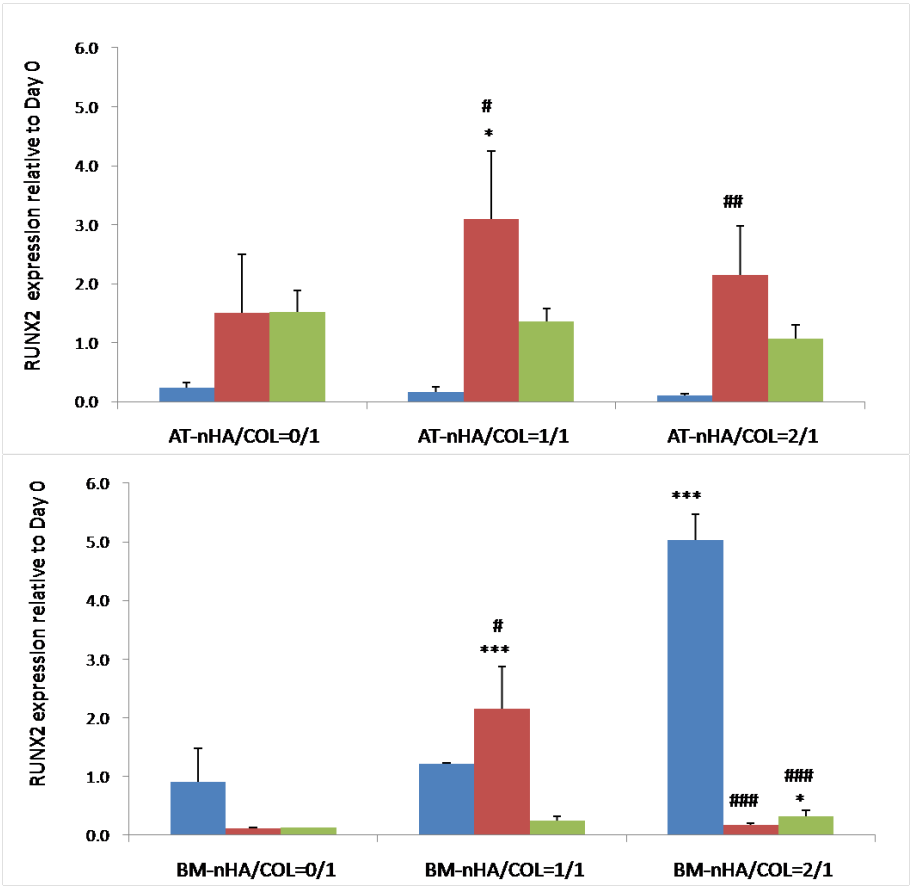

Figure 5.7: Gene expression profiles of RUNX2 (A) in AT-MSCs and (B) in BM-MSCs. * indicates significantly different compared to nHA/COL=0/1 control, at the same time point (p-values: *p <0.05, ** p<0.01, *** p<0.001), # indicates significantly different compared to day 7 (p-values: #p <0.05, ## p<0.01, ### p<0.001)

COL1: AT-MSCs showed an upregulation of COL1 expression starting at day 28 (Figure 5.9A), irrespective of construct type. Significantly higher COL1 expression (relative to day 14) was observed for AT-nHA/COL=0/1 at day 28 and 35 ($p< 0.001$), for AT-nHA/COL=1/1 at day 28 ($p< 0.01$) and 35 ($p< 0.001$), and for AT-nHA/COL=2/1 at day 28 ($p< 0.01$) and 35 ($p< 0.01$). Relative to AT-nHA/COL=0/1, the expression of COL1 was significantly higher for AT-nHA/COL=1/1 at day 35 ($p< 0.001$) and for AT-nHA/COL=2/1 at day 28 and 35 ($p< 0.01$). BM-MSCs showed high expres-

sion of COL1 at day 14, after which the COL1 expression was downregulated until the end of the culture period (Figure 5.9B). Relative to day 14, significantly lower COL1 expression was observed for BM-HA/COL=0/1 at day 28 ($p< 0.05$), for BM-HA/COL=1/1 at day 28 and 35 ($p< 0.01$) and for BM-HA/COL=2/1 at day 28 and 35 ($p< 0.01$). Relative to BM-HA/COL=0/1, significantly higher COL1 expression was for BM-HA/COL=1/1 at day 14 ($p< 0.01$) and for BM-HA/COL=2/1 at day 28 ($p< 0.01$).

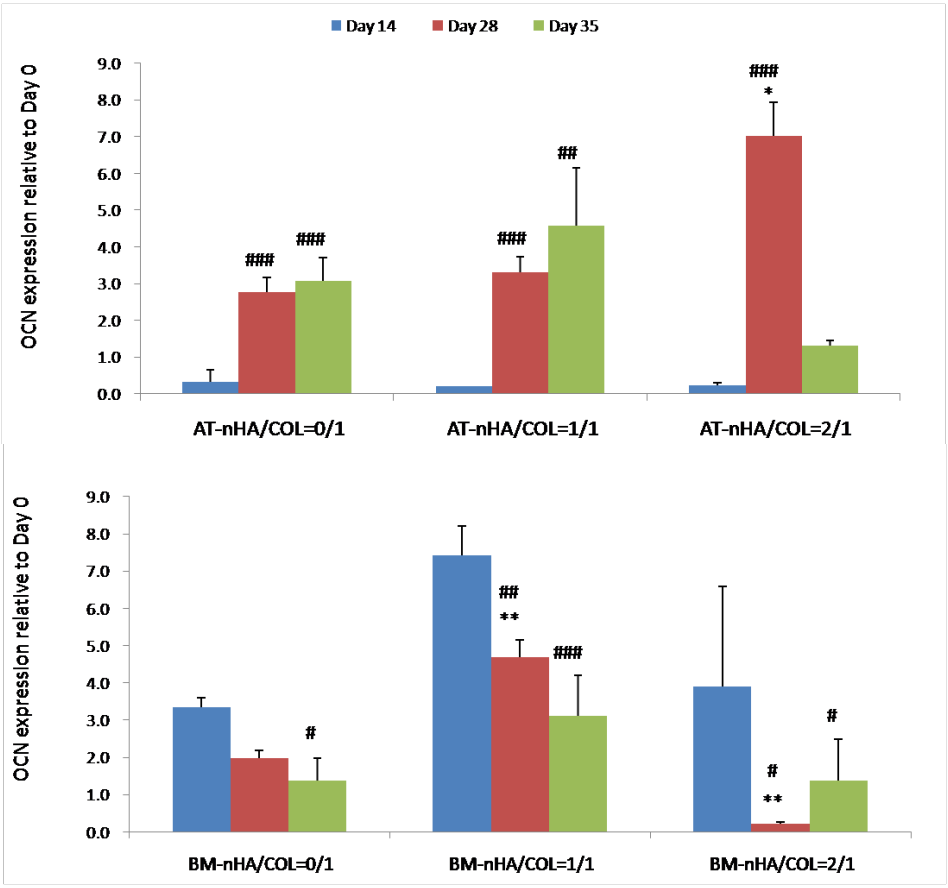

Figure 5.8: Gene expression profiles of OCN (A) in AT-MSCs and (B) in BM-MSCs. * indicates significantly different compared to nHA/COL=0/1 control, at the same time point (p-values: *p <0.05, ** p<0.01, *** p<0.001), # indicates significantly different compared to day 7 (p-values: #p <0.05, ## p<0.01, ### p<0.001)

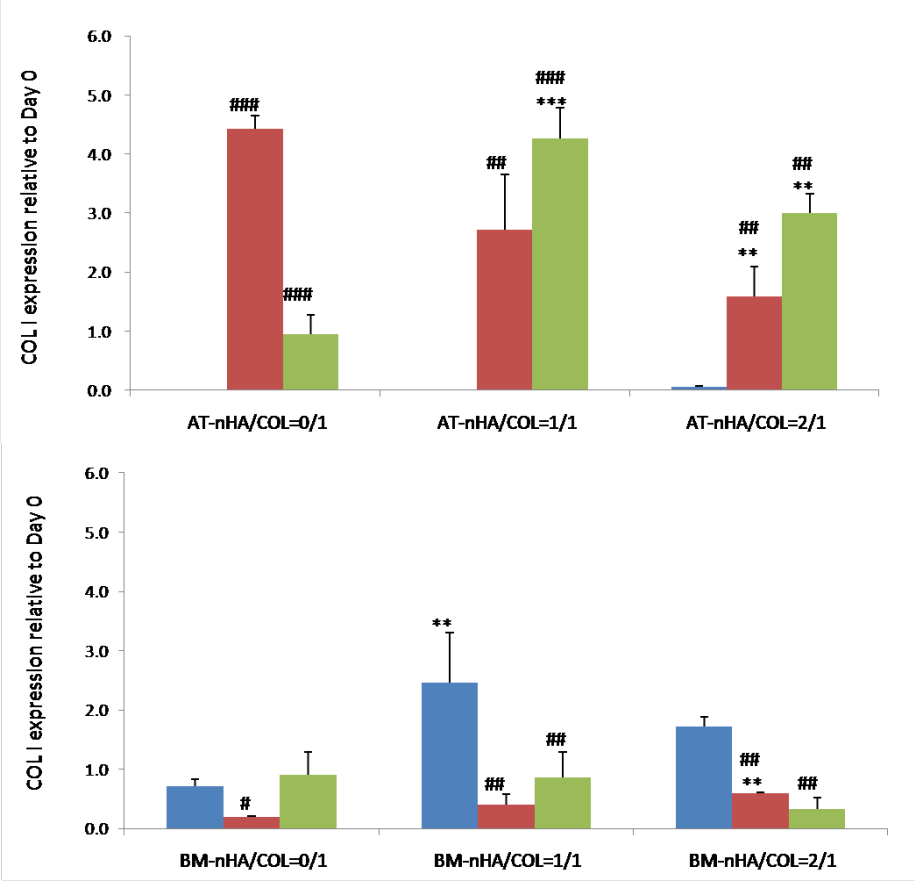

Figure 5.9: Gene expression profiles of COL1 (A) in AT-MSCs and (B) in BM-MSCs. * indicates significantly different compared to nHA/COL=0/1 control, at the same time point (p-values: *p <0.05, ** p<0.01, *** p<0.001), # indicates significantly different compared to day 7 (p-values: #p <0.05, ## p<0.01, ### p<0.001)

5.3.3.1 Histological analysis & Immunohistochemistry

HE stain: HE-stained histological sections of all experimental construct as well as HE stained sections of human bone (positive control) are presented in (Figure 5.10A).

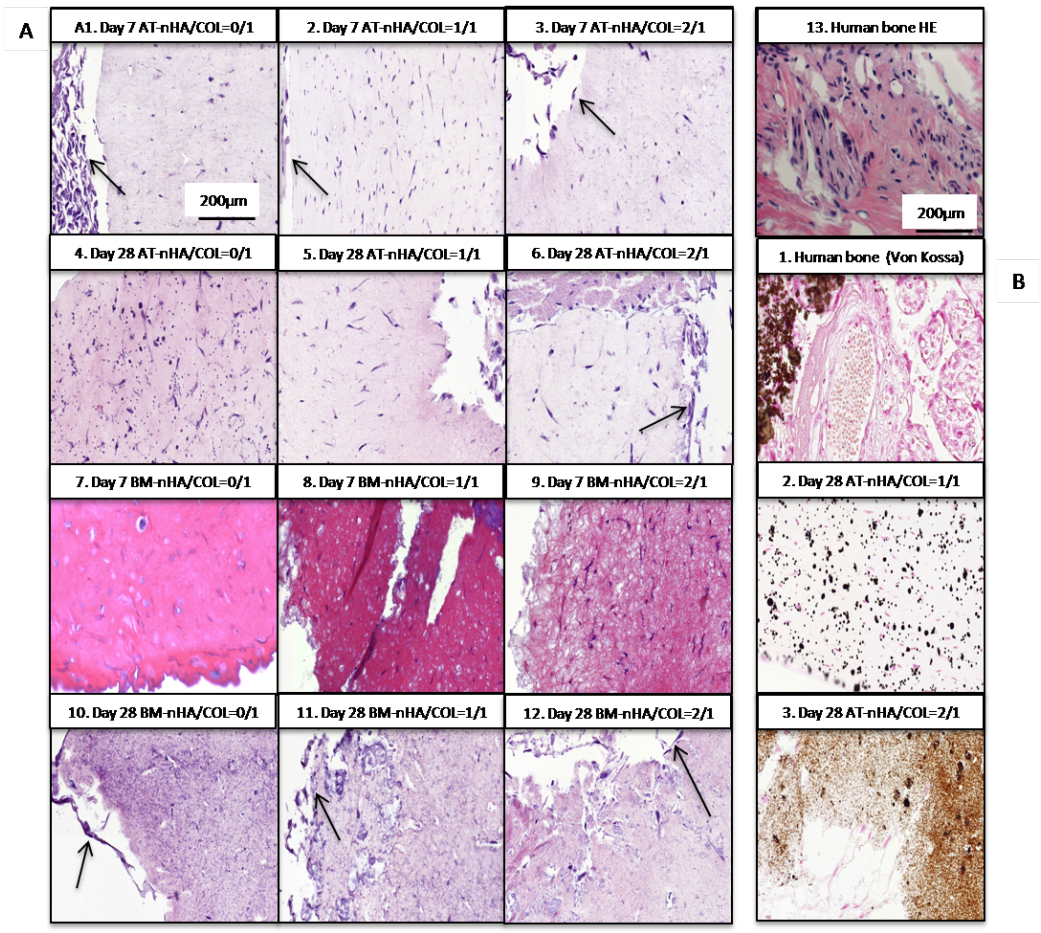

Figure 5.10: (A) HA staining of different group of nHA/COL constructs after 7 and 28 days of culture, (B) Von Kossa staining of different group of nHA/COL constructs after 28 days of culture.

Results of AT-MSC constructs showed that at day 7 as well as at day 28, cells were distributed throughout the entire construct. At the periphery of constructs, the cellular density was apparently higher compared to the centre of constructs (high density cell populations are indicated with arrows in Figure 5.10A 1, 2, 3, 5). The distribution of BM-MSCs in nHA/COL constructs was different at different time points. At day 7, cells were homogeneously

distributed throughout the entire construct (Figure 5.10A-7, 8, 9). However, at day 28 cells were mainly located at the periphery of constructs, and only few cells were detectable in the central region (Figure 5.10A-10, 11, 12). **Von Kossa stain**: To monitor the mineral deposition inside the constructs, Von Kossa staining was used (Figure 5.10B). Mineral deposition was only detectable for AT-nHA/COL=1/1 and AT-nHA/COL=2/1 at day 28.

IHC-OCN stain: AT-MSCs showed detectable OCN expression at day 28, especially for AT-nHA/COL=0/1 (Figure 5.11- 4, 5, 6).

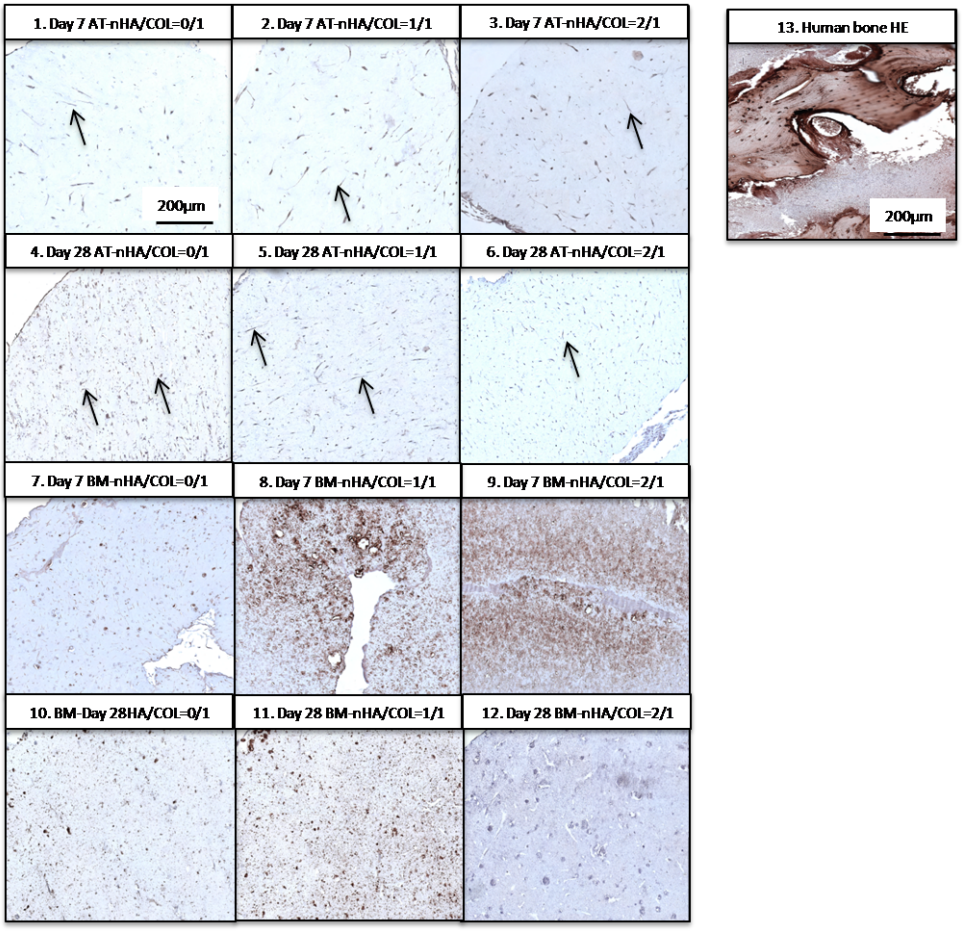

Figure 5.11: Immunohistochemical staining of OCN, in different group of nHA/COL constructs, after 7 and 28 days of culture

OCN protein was homogeneously distributed throughout the entire construct. AT-MSCs were observed in these constructs with an elongated, spindle-shaped morphology (highlighted with arrows in Figure 5.11-4, 5, 6). BM-MSCs showed high OCN expression at day 7, especially for BM-nHA/COL=1/1 and for BM-nHA/COL=2/1 (Figure 5.7-8, 9). At day 28, the OCN was still detectable for BM-nHA/COL=0/1 and BM-nHA/COL=1/1, but not for BM-nHA/COL=2/1 (Figure 5.11-10, 11, 12).

5.4 Discussion & Conclusions

The aim of this study was to comparatively evaluate the *in vitro* effect of biomimetic nHA/collagen based composite hydrogels (with different concentrations of nHA) on the behavior of MSCs, isolated from adipose tissue (AT-MSCs) and bone marrow (BM-MSCs). We hypothesized that (i) nHA/collagen based hydrogels promote the osteogenic differentiation of MSCs in nHA concentration dependent manner, and that (ii) AT-MSCs will show higher osteogenic capacities compared to BM-MSCs, because of their intrinsic proliferation and osteogenic differentiation potential in 2D *in vitro* cultures. This study indicated that AT-MSCs show high proliferation, differentiation and mineralization in nHA/COL constructs, irrespective of nHA concentration, whereas BM-MSCs showed only marginal cell proliferation and osteogenic differentiation in all experimental nHA/COL constructs. Based on the results of ALP activity and OCN protein level, the osteogenic differentiation of BM-MSCs started in the beginning of the culture period and for AT-MSCs at the end of the culture period. At a molecular level, both cell types showed high expression of osteogenic markers (BMP2, RUNX2, OCN or COL1) in both a nHA concentration and time dependent manner.

At a cellular level, the material properties (different nHA/COL ratio) did not show any remarkable effect on cellular behavior of AT-MSCs or BM-MSCs.

However, apparent differences were observed between cell types, i.e. the source for MSCs. AT-MSCs showed higher proliferation and osteogenic differentiation compared to BM-MSCs. This phenomenon corroborates several earlier studies [21, 23], but this study demonstrates that the cellular behavior of AT-MSCs does not change in a nHA concentration dependent manner. High proliferation levels of AT-MSCs could potentially provide faster cell-cell interaction compared to BM-MSCs, and this cellular interaction in 3D micro-environment could promote subsequent AT-MSC osteogenic differentiation [23, 31]. Remarkably, BM-MSCs started to proliferate and differentiate already within the first week of culture, irrespective of nHA concentration, whereas AT-MSCs started to differentiate only after the third week of culture. Early osteogenic differentiation of BM-MSCs can be explained with faster osteocyte formation. The signaling mechanisms involved in osteocyte formation could be activated at different time points in AT-MSCs and BM-MSCs, because AT-MSCs and BM-MSCs were cultured in different (PL or FBS supplemented) osteogenic media with different content of signaling molecules [32].

Molecular analysis of osteogenic markers, i.e. BMP2 (osteoblast differentiation marker), RUNX2 (essential element for osteogenic differentiation and skeletal morphogenesis), OCN (later stage osteogenic differentiation marker) and COL1 (crucial element of connective tissue) [33, 34, 35] showed that the material properties (nHA concentration) have an effect on the gene expression pattern of these molecules. It is known that nHA play a functional role in integrin-mediated cell adhesion and signaling [36]. Cell adhesion to ECM is mediated by transmembrane receptors [37]. This initiates intracellular signals, and focal adhesion kinase activation [38], which play crucial role in activation of downstream signaling. This signaling cascade can stimulate the activation of transcription factors and subsequent signal transduction [39]. For AT-MSCs, the expression of BMP2, RUNX2 and COL1 was promoted by AT-nHA/COL=1/1 constructs, whereas the expression of OCN and COL1 was promoted by AT-nHA/COL=2/1 constructs.

For BM-MSCs the expression pattern of BMP2, RUNX2, OCN and COL1 was remarkably higher in BM-nHA/COL=1/1 constructs, especially in the beginning of the culture period (at day 14). This observation proved that the osteogenic differentiation of BM-MSCs starts at an earlier time points compared to AT-MSCs. Moreover, already at day 7, BM-MSCs expressed high levels of OCN, which also indicates early osteogenic differentiation of BM-MSCs. Next, AT-MSCs and BM-MSCs showed a similar pattern of BMP2 expression. It has been shown that AT-MSCs can respond to BMP2 signals and express other osteogenic markers (e.g. OPN and RUNX2) [40], which indicates that AT-MSCs are a reliable cell type for future use in cell-based bone regenerative strategies.

In conclusion, AT-MSCs demonstrated higher osteogenic potential in nHA /COL based 3D micro-environments compared to BM-MSCs, in which proliferation and osteogenic differentiation were highly promoted. The proliferation and osteogenic differentiation pattern of AT-MSCs and BM-MSCs was regulated in a time dependent manner, irrespective of nHA amount in the constructs. On the other hand, nHA/COL ratios differently affected gene expression profiles of AT-MSCs and BM-MSCs. The fact that AT-MSCs showed high proliferation and mineralization is appealing for their application in future (pre-)clinical research as an alternative cell source for MSCs.

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CHAPTER 6

Effect of Calcium Phosphate Ceramic Substrate Geometry on Mesenchymal Stromal Cell Organization & Osteogenic Differentiation

6.1 Introduction

One of the most challenging aspects in tissue regenerative research is to find an appropriate material for bone regeneration therapy. Because of high osteoinductive and osteoconductive capacities and hence clinical efficacy, autologous bone grafting is still the most frequently applied material for bone regenerative treatments [1]. However, bone grafting is an invasive procedure, associated with high morbidity due to a second surgery site, and the often limited amount of available bone [2]. In view of this, it has become a scientific challenge to find effective alternatives for autologous bone grafts. Calcium phosphate (CaP) based biomaterials have become the most frequently utilized synthetic alternatives for autologous bone, for their chemical composition strongly resembling the inorganic part of bone tissue [3],

and their high bioactive and osteoconductive capacity [4]. Multiple *in vitro* studies showed that CaP ceramics (e.g. HA and tricalciumphosphate, TCP) promote proliferation and osteogenic differentiation already at the cellular level [5, 6]. The efficacy of CaP is nowadays also exploited for clinical application as bioactive coatings for the surface of new generation implants. Such coatings were shown to stimulate osseointegration resulting in firm fixation between implant and bone [7]. Another factor that is known to play a crucial role for osseointegration is surface topography [8, 9]. Several studies have indicated that micro- and nano-scale surface topography of CaP ceramics strongly influences cell adhesion, proliferation and differentiation [10, 11, 12]. By combining topographical and chemical properties of implants, several studies have investigated the osteoinductive effect of (repetitive) surface concavities on bulk CaP ceramics and CaP-coated titanium implants [13, 14, 15]. The authors demonstrated that the presence of surface concavities can provide osteoinductive capacity of the ceramic material, concluding that topographical and chemical properties of implants can mutually steer biological responses and (bone) tissue formation *in vivo* [13]. We recently studied the concept of mineralization within surface concavities and showed that cell-independent mineralization in simulated body fluid is strongly controlled by concavity dimensions [16]. Among the concavities in different CaP ceramics, the mineralization process started preferentially in small concavities ($\sim$440 μm hemispheres) of hydroxyapatite (HA) disks sintered at 1200°C, where the mineralization was increased 124-fold compared to medium (800 m hemispheres), and 10-fold compared to large concavities ($\sim$ 1800 μm hemispheres) [16]. It is known that physical factors, such as geometrical properties of surface, mechanical forces, and extracellular matrix texture, have strong influence on MSC behavior. In response to extracellular stimulation, cells activate their internal signaling pathways and subsequent mechano-transduction, which both control MSC behavior [17]. However, experimentally little is known about the behavior of MSCs, cultured on pure HA based scaffolds, which are covered with different size surface concavities.

To evaluate the effect of surface concavities of CaP ceramics on cellular behavior of MSCs, human adipose tissue derived mesenchymal stromal cells (AT-MSCs) were cultured on CaP ceramic scaffolds with different surface-concavity sizes. The outcome parameters included analyses on cellular organization, cell proliferation, and osteogenic differentiation. We hypothesized that compared to planar controls, surface concavities would promote cell proliferation, cellular organization within the concavities, and osteogenic differentiation of cells, because of mineral (CaP) nucleation in concavities and a more 3D microenvironment [16]. Additionally, we hypothesized that MSC proliferation and osteogenic differentiation would increase with smaller concavity size due to more rapidly occurring 3D cell-cell interactions and subsequent molecular cross-talk.

6.2 Materials & Methods

6.2.1 Substrate preparation & characteristics of disks

HA powder (Merck, Darmstadt, Germany) with a Ca/P molar ratio of 1.67 was used in this study. The powder was uniaxially pressed at 103 MPa (15,000 psi) for 10 min in a cylindrical steel mold (internal diameter $\sim$ 21 mm). Subsequently, cylindrical HA ceramics were heat-treated in a furnace at 800°C for 6 h (1.67°C min^{-1}) to provide suitable strength to the ceramic to withstand the stresses applied during the machining process. Thereafter, heat-treated HA cylinders were cut into disks (thickness 4 mm, diameter 21 mm). Drill tips with different diameter sizes (2.1, 1.0 and 0.5 mm; Horico Dental, Berlin, Germany) were used to prepare hemispherical concavities at the disk surfaces. Subsequently, the disks were sintered in a furnace for 6 h (1.67°C min^{-1}) at 1200°C, by which disk dimensions were reduced to 3 mm thickness and 16 mm diameter and cavities sizes to diameters of 440 μm (small concavity; SC), 800 μm (medium concavity; MC) and 1800 μm

(large concavity; LC). Afterwards, the disks were prepared based on the experimental setup.

For imaging, the heat-treated HA disks were divided (without cutting) in three different regions, each containing a specific cavity size/diameter (SC $\rightarrow$ 440 μm, MC $\rightarrow$ 800 μm or LC $\rightarrow$ 1800 μm) with total of 6 cavities in each size. The flat surface in the center of the disk was used as a planar control (Figure 6.1A).

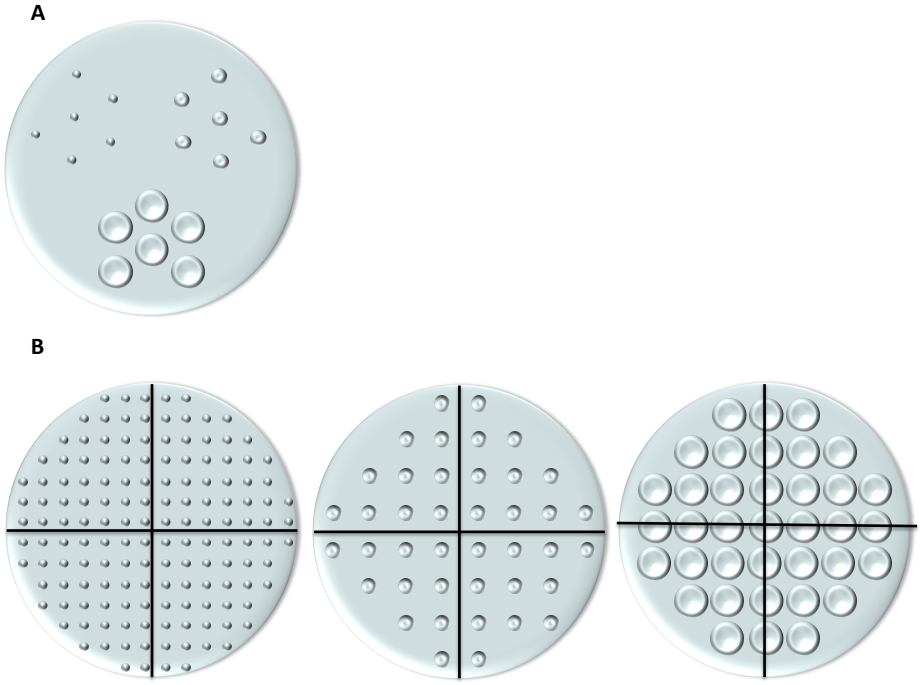

Figure 6.1: Schematic representation of disk surface topography employed in the study. (A) Schematic distribution of the concavities at the surface of the disks for confocal microscopy; 6 concavities of each size (small cavity (SC)'440 m, medium cavity (MC)' 800 m; and large cavity (LC)' 1800 m) were prepared on the surface of the disks, and the center to center distance was kept the same (SC=2.1, MC=2.5, LC=2.8 mm). The planar surface in the centre of the disk was considered as a control planar surface (PS). (B) Schematic distribution of the concavities at the surface of the disks for biochemical assays. Disks were completely covered with each size of concavities (SC, MC, LC) center to center distances SC=0.6, MC=1.2 and LC=2.7 mm

For the evaluation of cellular behavior and gene expression, the heat-treated HA disks featuring concavities of the same size, were cut in 4 pieces (n=4, Figure 6.1B). The total number of hemispherical concavities in each disk was 148 for SC, 42 for MC, and 36 for LC, resulting in a total surface area of respectively 2.23 cm^2 (SC), 2.22 cm^2 (MC) and 2.92 cm^2 (LC). As a control group, planar disks (PS) were used with a total surface area of 2.01 cm^2 (Figure 6.1B). Before cell seeding, disks were autoclave-sterilized.

6.2.2 AT-MSC isolation, expansion & characterization

AT-MSCs were isolated from fat tissue of healthy donors (Department of Plastic Surgery, Radboudumc, Nijmegen, the Netherlands) after written informed consent, following a previously described AT-MSCs isolation procedure [18, 19]. Harvested cells were cultured in proliferation medium, consisting of alpha Minimal Essential Medium (α-MEM; Gibco®, Life Technologies, Grand Island, USA) supplemented with 5% platelet lysate (PL, (Sanquin Blood Bank, Nijmegen, the Netherlands), pooled from 5 different donors, 100 U/ml penicillin (Gibco), 10 g/ml streptomycin (Gibco®), and 10 U/ml heparin (LEO Pharma, Amsterdam, the Netherlands) at 36°C in a humid atmosphere with 5% CO2. Medium was changed twice a week. Cells were passaged upon reaching $\sim$ 80% confluency using 0.25% w/v trypsin/0.02% EDTA (Gibco®). Prior to MSC characterisation, harvested cells, from the first passage, were examined by fluorescence-activated cell sorting (FACS) for positive expression of CD73, CD90 and CD105 (eBioscience, San Diego, USA) and negative expression of CD45 (R& D system, Abingdon, United Kingdom). Next, MSCs from passage 3 were examined with three biochemical assays i.e. DNA assay for proliferation capacity, and osteogenic differentiation and Ca deposition assay for later stage of osteogenic differentiation. The cells from passage 4 were used for this study. The experiment was repeated three times.

6.2.3 Biochemical assay for cellular DNA content

AT-MSCs were cultured on disks, using 10.000 cells/cm^2 as the seeding density (relative to PS). Cells were cultured in the osteogenic medium consisting of α-MEM supplemented with 5% PL, 100 U/ml penicillin, 10 μg/ml streptomycin, 10 U/ml heparin, 0.2 mM L-ascorbic acid-2-phosphate (Sigma-Aldrich, St. Louis, MO, USA), 2mM L-glutamine (Gibco®), 10^{-8}M dexamethasone (Sigma-Aldrich) and 0.01 M β-glycerophosphate (Sigma-Aldrich), at 37°C in a humid atmosphere with 5% CO_2. Cell proliferation was evaluated with a DNA assay. Samples were collected at 3 time points relative to cell seeding (i.e. day 3, 14, 28). Prior to sample preparation, cell layers were washed twice with PBS, 1 ml MilliQ was added, and the sample was stored at -80°C. After two repetitive freeze/thaw cycles, samples were used for DNA assay. The cellular DNA content was measured using a QuantiFluor® dsDNA System Kit (Promega, Madison, Wisconsin, USA). For the standard curve, serial dilutions of dsDNA stock (range: 0-2000 ng/ml) were prepared. Next, 100 μl of either sample of standard solution was added into the wells, followed by 100 l of working solution. The plate was incubated at room temperature for 5 min, and then the absorbance of samples/standards was measured at 504 nm excitation and 541 nm emission, using a fluorescence microplate reader (FL600, BioTek, Canada).

6.2.4 Immunofluorescence & quantitative cell measurements

For the immunofluorescence and quantitative cell measurements, at selected time points (3, 14, 21 and 28 days), samples were collected by washing 2x in phosphate buffered saline (PBS, Gibco®), fixation in 2% paraformaldehyde (PFA, Sigma-Aldrich) for 20 min, permeabilization in PBS containing 0.25% Triton X100 (Koch, Light-Laboratories, Colebrook, UK) for 10 min, washed

again 2x in PBS and blocking with 1% BSA (Sigma Aldrich) for 1 hour. Thereafter, cells were washed 2x in PBS and stained as follows:

1. For visualization of nuclear dsDNA, cells were stained with DAPI (Invitrogen, Molecular probes) for 1 min, in a dilution of 1:2500. Afterwards, cells were washed 2 x 5 min in PBS,

2. For visualization of cytoskeletal morphology, the filamentous F-actin was stained with Alexa Flour® 568 phalloidin (Invitrogen, Molecular probes, Eugene-Oregon, USA) for 2 hours, in a dilution of 1:250. Afterwards cells were washed 2 x 5 min in PBS,

3. For visualization of the osteogenic marker osteocalcin, cells were stained with a monoclonal osteocalcin antibody conjugated with Alexa Flour® 488 (Novus Biologicals, Cambridge, UK) for 2 hours in a dilution of 1:1000. Afterwards, cells were washed 2 x 5 min in PBS.

Subsequently, the samples were mounted with Mowiol® 4.88 solution (Merk Millipore, Darmstadt, Germany), covered with cover-glasses, and stored at +4°C for fluorescent imaging.

For visualization of calcium embedded within the extracellular matrix, at the same time points (3, 14, 21 and 28 days), separate samples were stained with 2% w/v Alizarin Red S 598 (ScienCell Research Laboratories, Carlsbad CA, USA) for 15 min. Subsequently, samples were washed 5x with PBS for 1 min and stained with DAPI for 1min in a dilution 1:2500. Afterwards, the samples were washed and mounted with Mowiol® 4.88 solution (Merk Millipore, Darmstadt, Germany), covered with cover glasses, and stored at +4°C for fluorescent imaging.

The substrates were analyzed with a fluorescence microscope (Axio Imager Microscope Z1, Carl Zeiss Micro imaging, Gottingen, Germany). The excitation and emission wavelength for a filamentous F-actin was 568 and 603

nm, for dsDNA (DAPI) 405 and 428 nm, for osteocalcin 495 and 519 nm, and for alizarin red 590 and 617 nm, respectively. The images were captured with a 20x/0.75 objective.

To obtain high-resolution 3D images for analysis of cellular organization within the concavities, the samples (day 3, 14, 28) were analyzed by confocal laser scanning microscopy (CLSM; Olympus FV1000, Olympus, Tokyo Japan). The images were captured with 20x/0.75 objective. In order to obtain qualitative and quantitative data on concavity volumes, the images were analyzed with ImageJ imaging software (Centre for Information Technology, National institutes of Health, Bethesda MD, USA), using 3D object counter plugin. For quantification of cell nuclei, an average of single cell immunofluorescence intensity was measured (from the samples at day 3) and this average intensity level was considered as a standard for cell quantification.

6.2.5 RNA isolation & reverse transcription

For RNA isolation, separate samples, collected at day 3, 14, 28, were washed 2x with PBS, treated 500 μl 'RNA' lysis buffer from Genelute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich), and stored at -800°C until RNA extraction. RNA isolation was performed according to manufacturer instructions. The extracted RNA was quantified with a spectrophotometer (Nanodrop Technologies, Wilmington, DE). For reverse transcription (cDNA synthesis), an iScript^{TM} cDNA kit was used (Bio-Rad, Hercules, CA, USA). For each cDNA reaction, 15 μl RNAse free water containing 1 μg RNA, mixed with 4μl 5xiScript reaction mix and 1μl reverse transcriptase was used. The samples (cDNAs) were stored at -20°C until use. Before use of samples (cDNAs), they were diluted 5 times by adding 80 μl of nuclease free water.

6.2.6 Real-time polymerase chain reaction

For real-time polymerase chain reaction (RT-PCR), qPCR Master Mix Plus/ SYBR Green I (Eurogentec, Seraing, Belgium) was used according to the manufacturer's instructions. Briefly, for each reaction 12.5 μl SYBR Green Supermix, 3 μl of primer mix, 4.5 μl of nuclease free water and 5 μl diluted cDNA was used. RT-PCR was completed with 40 amplification cycles. The genes of interest were Osteocalcin (OCN), Bone Sialoprotein (BSP), Bone Morphogenetic Protein 2 (BMP2), Runt-related Transcription Factor 2 (RUNX2), and Collagen type 1 (COL1). The sequence of applied primers is given in Table 6.1. The raw data for respective transcripts were normalized to expression of 60S Acidic Ribosomal Protein P0 (RPLP0) within the same sample/RNA. The gene expression and fold changes were calculated according to Livak & Schmittgen ($2^{-\Delta\Delta C_t}$) relative to control samples (collected from PS) [20].

6.2.7 Statistical analysis

Data are presented as mean ± standard deviation. Statistical analysis was performed based on N=3 (for all experimental groups) with Graphpad Prism® 5.03 software (Graphpad Software Inc, San Diego, CA, USA). Quantitative results were analyzed using a one-way ANOVA with a posthoc Tukey Multiple Comparisons Test. A probability (p) value < 0.05 was considered statistically significant.

Table 6.1: Primer sequences for RT-PCR.

Gene name	Sequences
RPLP0	Forward- TTCTTCTTTGGGCTG GTCAT Reverse- TTGGGTAGCCAATCTGCAGA
RUNX2	Forward- TCTGGCCTTCCACTCTCAGT Reverse GACTGGCGGGGTGTAAGTAA
BMP-2	Forward- CCCAGCGTGAAAAGAGAGAC Reverse- GGAAGCAGCAACGCTAGAAG
Col type 1	Forward- GGTGTAAGCGGTGGTGGTTAT Reverse- AGGTTCCCCGTTCTCACTTT
OCN	Forward- GGCGCTACCTGTATCAATGG Reverse- GTGGTCAGCCAACTCGTCA
BSP	Forward- GACTGTGACGAGTTGGCTGA Reverse- AGGTTCCCCGTTCTCACTTT

6.3 Results

6.3.1 Cellular behavior

During the first 14 days, MSCs showed the highest proliferation on PS disks, and lowest proliferation on SC disk. However, after 28 days of culture, an increased cell proliferation was observed on SC and MC disks compared to PS disks (Figure 6.2a).Because of the differences in surface area of the disks (PS→2.01 cm^2, SC→2.23 cm^2, MC→2.22 cm^2 and LC→2.92 cm^2), the results of DNA content were normalized for surface area (Figure 6.2b). After normalization, MSC proliferation patterns remained similar, showing at day 14 a significantly lower DNA-content for SC ($p< 0.001$), MC ($p< 0.001$), and LC ($p< 0.05$), and at day 28 significantly higher DNA-content values for SC ($p< 0.01$) and MC ($p< 0.01$) compared to PS.

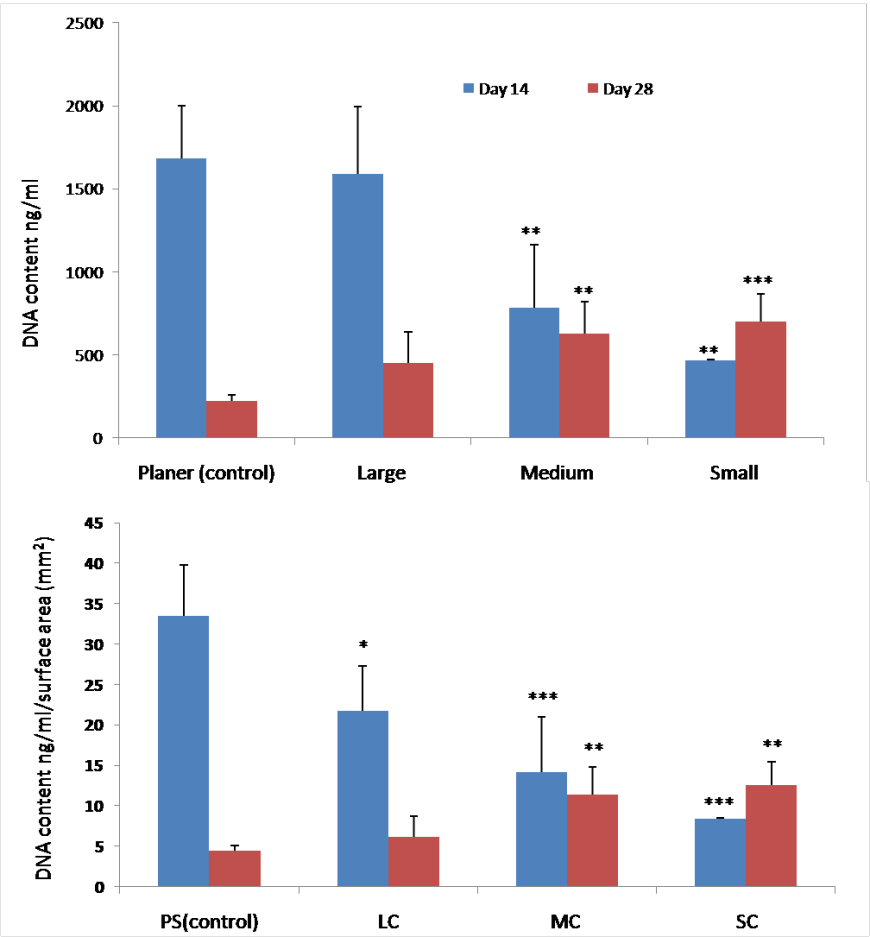

Figure 6.2: (a) Cellular DNA content/proliferation level during 28 days of culture. (b) DNA content normalized for surface area of the disks. (p-values: *p <0.05, ** p<0.01, *** p<0.001) compared to control planar surface (PS); indicate significant differences compared to PS control at the same time point. ND means not determined.

6.3.2 Cellular organization

6.3.2.1 Immunofluorescence results

The samples for immunofluorescence were stained with 3 different dyes, i.e. DAPI (in blue) for nuclear visualization, actin (in red) for visualization of the

cytoskeleton, and osteocalcin (in green) for late osteogenic differentiation. After each staining, the samples were observed by means of fluorescence microscopy (Figure 6.3).

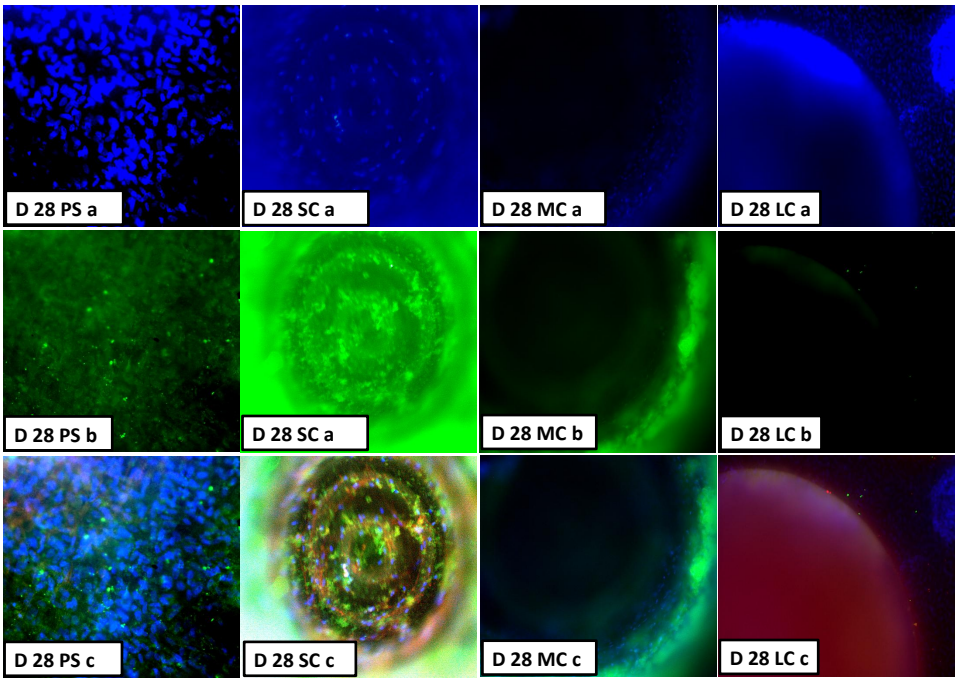

Figure 6.3: Fluorescence microscopy images. For visualization of dsDNA in nuclei, cells were stained with fluorescent DAPI (in blue); for cytoskeletal structure visualizations, filamentous F-actin (in cells) was stained with Alexa Flour 568 phalloidin (in red); for visualization of osteocalcin, cells were stained with a monoclonal osteocalcin antibody, labeled with Alexa Flour 488 (in green).

At day 28 osteocalcin-positive signal (green) was mainly detected in SC and MC. In SC, osteocalcin was distributed within the entire concavity volume, while in MC osteocalcin was only detected on the edges of the concavities (Figure 6.3). To visualize the mineralization pattern of AT-MSCs, separate samples were stained with Alizarin Red S 598 (Figure 6.4). During 28 days of culture, a gradual increase of calcium positive staining was observed on

the surface of the experimental scaffolds in presence of cells. Contrary, no positive Alizarin staining could be observed in the cell-free control at day 28.

Figure 6.4: Qualitative evaluation of calcium deposition during 28 days of culture. Samples were stained with Alizarin Red S 598 (in red)

6.3.2.2 Qualitative results of cellular organization

Confocal microscopy showed that after 3 days of culture only few cells were situated inside of the concavities independent of the size (Figure 6.5). However, several differences in cellular organization were detectable. In SC and MC, cells were distributed already in a 3D multi-layer configuration, while in LC cells generally covered the concavity walls (Figure 6.5).

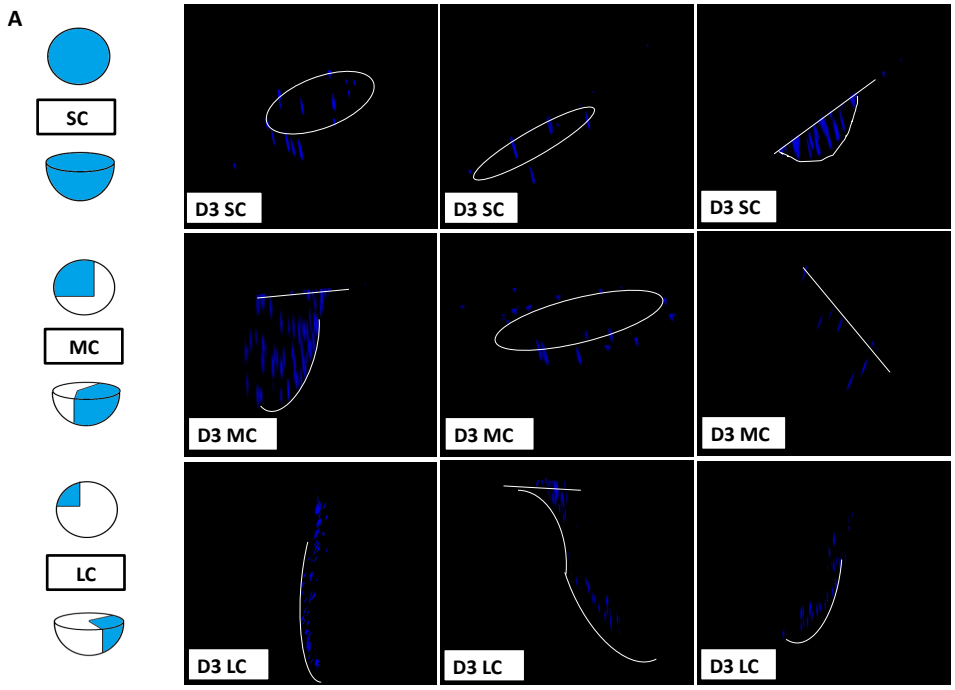

Figure 6.5: Confocal microscopy images with 3D reconstruction of SC, MC and LC. Cell nuclei were stained with fluorescent DAPI (in blue). Samples with different concavity sizes after 3 days of culture.

Cell nuclei in the Figure 6.5 present an elongated shape due to the fact that cells were viewed in a 3D environment and different angles were chosen (Image J) for visualization. After 14 days of culture, for all concavity sizes a substantial increase in the number of cell nuclei was observed. In SC and MC, cells created an apparent multilayered cell sheet, covering the walls, and in SC cells filling up the entire concavity volume (3D cellular organization). The planar surface between concavities was also covered with a cellular multilayer. In contrast to SC and MC, the cellular organization in LC was different, day 14, still a gap between these two cell populations in LC was observed (Figure 6.6).

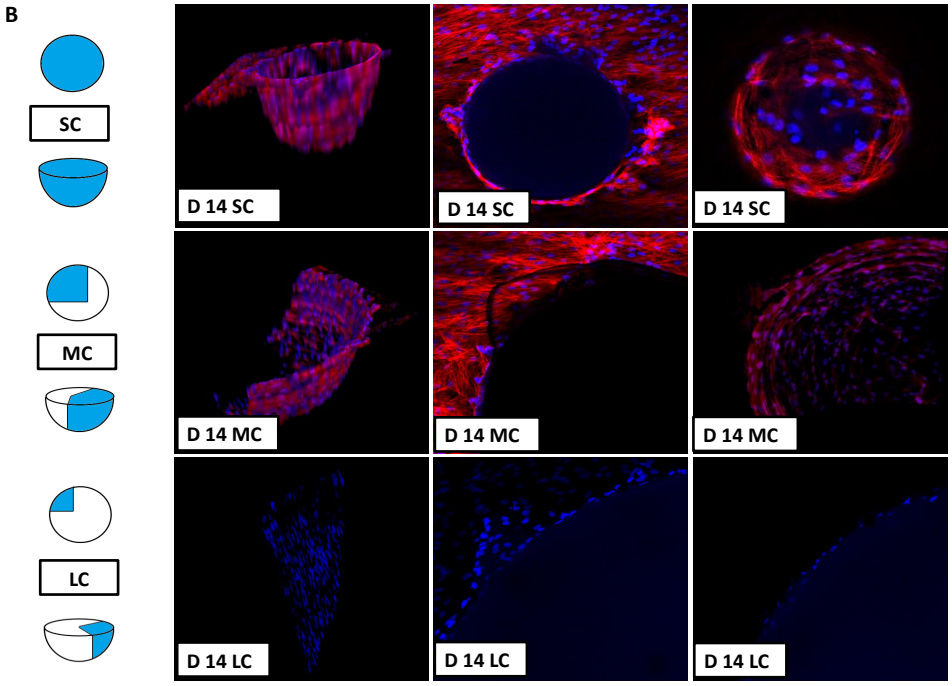

Figure 6.6: Confocal microscopy images with 3D reconstruction of SC, MC and LC. Cell nuclei were stained with fluorescent DAPI (in blue); filamentous F-actin (in cells) was stained with Alexa Flour 568 phalloidin (in red). Samples with different concavity sizes after 14 days of culture.

After 21 days of culture, very densely distributed cell nuclei were observed in SC and MC. In SC, cells homogenously distributed within the entire concavity volume. In MC, the cellular organization was different from SC with a less dense cell population in the center compared to the edges. The cellular organization in LC at day 21 was similar to the LC at day 14 with a separation in two cell populations (on the concavity walls close to surface area of the disk and at the bottom of the concavities), and still a gap between those populations (Figure 6.7).

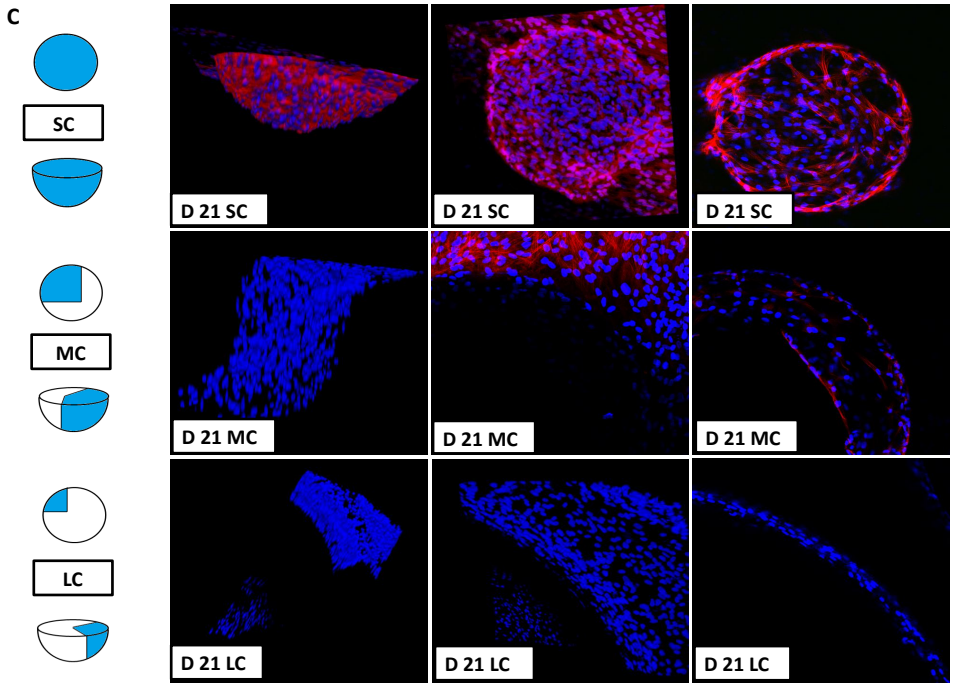

Figure 6.7: Confocal microscopy images with 3D reconstruction of SC, MC and LC. Cell nuclei were stained with fluorescent DAPI (in blue); filamentous F-actin (in cells) was stained with Alexa Flour 568 phalloidin (in red). Samples with different concavity sizes after 21 days of culture.

After 28 days of culture, cells in SC and MC were homogenously distributed within the entire concavity volumes. At day 28 in SC and MC cells already reached the highest deepness within the concavity volume. However, in LC the cellular organization was still different from SC and MC, with still the existence of two separated cell populations, but the distance between those populations was smaller compared to day 21 (Figure 6.8).

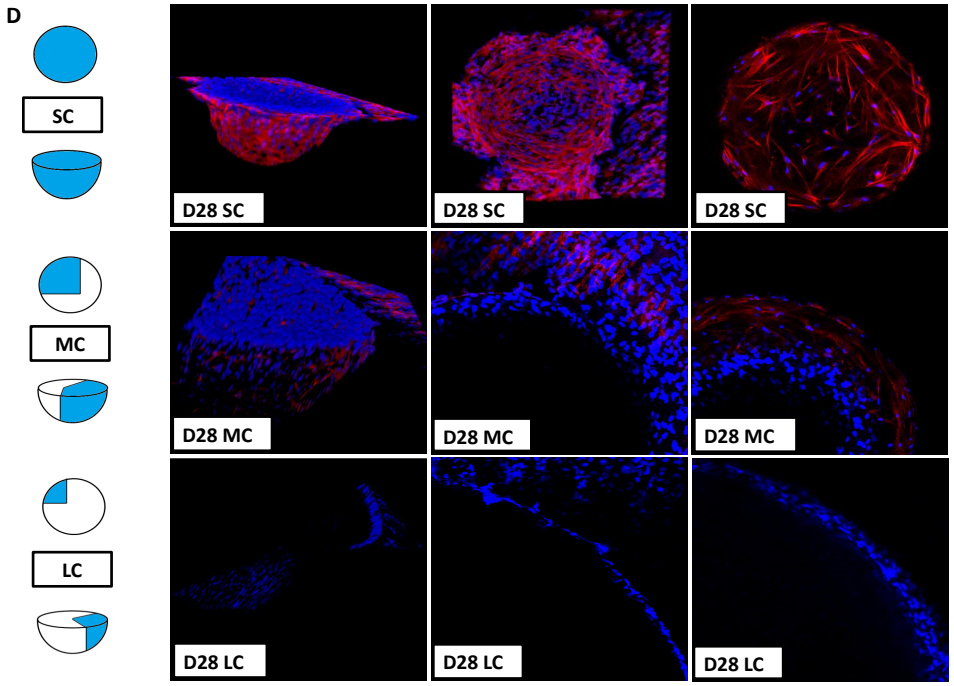

Figure 6.8: Confocal microscopy images with 3D reconstruction of SC, MC and LC. Cell nuclei were stained with fluorescent DAPI (in blue); filamentous F-actin (in cells) was stained with Alexa Flour 568 phalloidin (in red).Samples with different concavity sizes after 28 days of culture.

6.3.2.3 Quantitative results of cellular organization

Cell nuclei were quantified in PS, SC, MC and LC at all time points (Figure 6.9). The number of nuclei for SC, MC and LC was normalized for the concavity volume and the number of the nuclei for PS was normalized with the volume of one layer (L=0.75; B=0.75; H=0.01mm). In the first 3 days no significant differences were found in SC, MC, and LC compared to PS control. After 14 days, an increase in cell nuclei was observed for all groups, with the highest number of cell nuclei in PS. At day 14, the number of cell nuclei was significantly lower in SC ($p< 0.05$), MC ($p< 0.01$), and LC ($p< 0.01$)

compared to PS. At day 28 the number of cell nuclei was significant high in SC (p< 0.001) and in MC (p¡0.05) compared to PS. Cell nuclei numbers at day 28 in LC could not be determined due to large data information and software limitations.

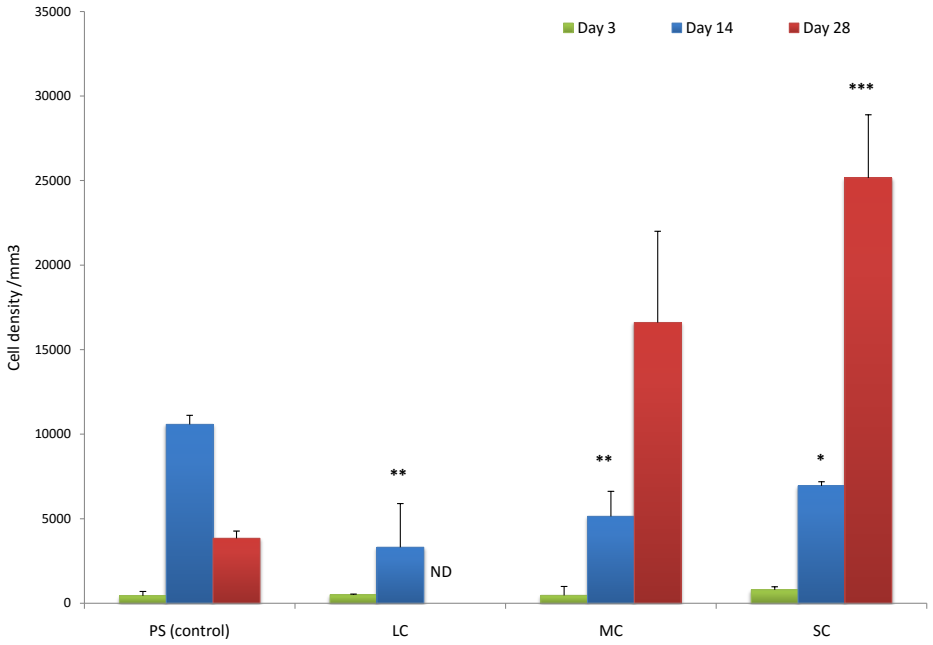

Figure 6.9: Results of quantitative analysis of the number of cell nuclei within the concavities normalized for concavity volume. P-values (p-values: *p <0.05, ** p<0.01, *** p<0.001) indicate significant differences compared to PS control at the same time point. ND means not determined.

6.3.3 Gene expression profile of selected molecules

To determine gene expression of selected genes, RT-PCR analysis was performed only at day 28 of culture. The samples collected at day 3, and 14 were

excluded from analyses because of insufficient extracted RNA. From selected molecules OCN expression showed an upregulation (up to ∼2.2 fold) in SC compared to PS control (p=0.0181). BMP2 was also upregulated in LC (up to 3 fold) compared to PS control (P=0.0088). Similar gene expression levels were observed for BSP, RUNX2 and COL1 (p> 0.05) (Figure6.10).

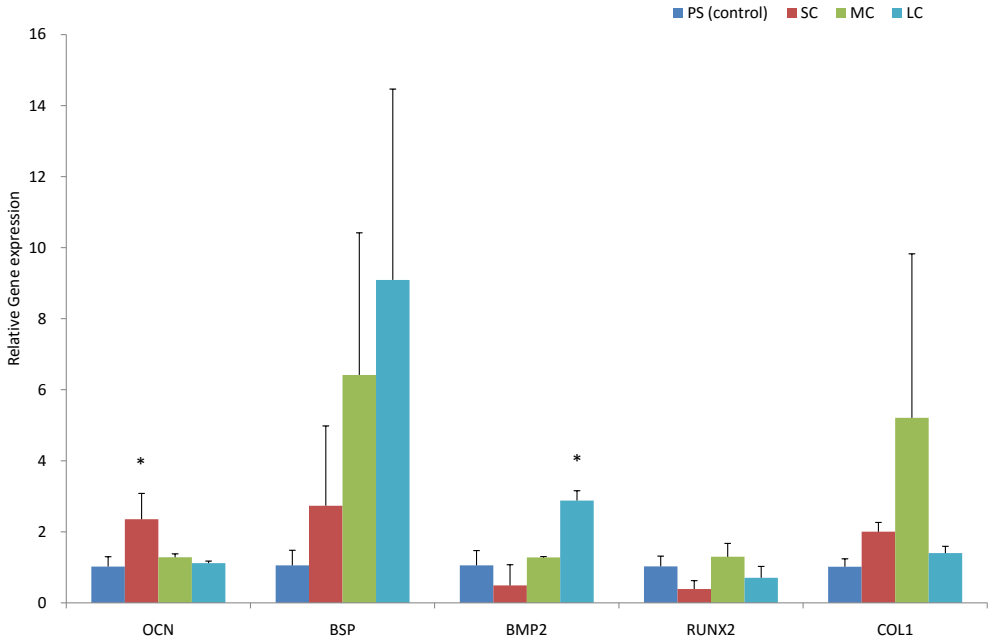

Figure 6.10: Gene expression of OCN, BSP, BMP2, RUNX2 and COL1 genes at 28 days of culture. * indicates significant different value compared to PS control (p-values: *p <0.05)

6.4 Discussion & Conclusions

The aim of this chapter was to evaluate the effect of surface concavities on CaP ceramic scaffolds on mesenchymal stromal cell organization, proliferation, and osteogenic differentiation. We hypothesized that compared to planar control surfaces, surface concavities would promote cell proliferation and cellular organization within the concavities and osteogenic differentiation of cells, because of a 3D microenvironment and mineral (i.e. CaP) nucleation in concavities [16]. Additionally, we hypothesized that MSC proliferation and osteogenic differentiation would increase with smaller concavity size due to more rapidly occurring 3D cell-cell interactions and subsequent molecular cross-talk. The main findings of this study were that surface concavities promote cell proliferation, in a concavity size dependent manner. At day 28, SC increased cell proliferation to a larger extent compared to MC and LC. Next, different size concavities regulated the distribution of cells and 2D/3D cellular organization. Finally, the concavity size influenced osteogenic differentiation of cells, showing an upregulation of OCN expression in SC (mRNA level) and OCN protein product detected in SC and MC.

Our study indicated that surface concavities promote cell proliferation, which was proven by quantification of both integral cellular DNA content and 3D cell nuclei. In addition, the proliferation pattern promoted by different concavity sizes was different. In SC, proliferation increased to a larger extent, because at day 28 the highest cell number (nuclei) and the highest amount of DNA content was found in SC. The quantitative analysis of cell nuclei showed that cell proliferation can be increased inside the concavities compared to PS and that the proliferation potential of cells is high inside SC, possibly due to cell proximity and faster cellular communication [21], because the cell-cell interaction and cellular communication is based on exchange of secreted molecules, which can activate appropriate signals in cells [22, 23]. In addition, cells are able to adhere to the surface, recognize its geometrical

properties, and respond to geometrical stimuli [24, 25]. This phenomenon is called adhesion-mediated signaling, which provides cells information about extracellular microenvironment, including geometrical and chemical characteristics of the substrate [26]. Based on the proliferation and differentiation results, cells seem to recognize the geometrical properties of the surface.

The fact that CaP-based biomaterials with specific surface geometry can promote bone formation *in vivo* was proven by others [27, 28, 29, 30]. However, little is known about the influence of CaP-based biomaterials combined with geometrical properties (e.g. size, shape) on cellular organization and cell differentiation [11]. In this study, we found that concavity size can influence cellular organization, because already on day 3 cells were distributed in a 3D-like manner in SC and MC, whereas in LC cells were distributed still as a monolayer. This observation can be explained by the fact that cells recognize geometrical properties of the surface and respond differently to their cellular organization ([24, 25]). Next, mineralization results of cells on the surface of experimental scaffolds (featuring different size concavities) showed a gradual increase in Ca deposition, which indicates that cells can proliferate as well as differentiate on the surface of CaP-based biomaterials. However, the method to visualize Ca deposition (Alizarin Red S staining) was not suitable for the quantification of mineralization within different concavity sizes. Therefore, for the analysis of osteogenic differentiation/mineralization within the concavities, the secretion of several osteogenic markers, i.e. osteocalcin (osteogenic differentiation marker), BSP (component of mineralized tissue), BMP2 (osteoblast differentiation marker) and RUNX2 (essential element for osteogenic differentiation and skeletal morphogenesis), were analyzed ([31, 32, 33]). We observed that concavity size influences osteogenic differentiation of MSCs, because higher levels of OCN protein (OCN immunostaining) were found in SC and MC compared to LC and PS. Moreover, RT-PCR results showed that OCN was overexpressed in SC compared to PS. These results indicate that extracellular stimulation factors (e.g. geometrical properties, cell-cell communication, and local calcium nucleation)

can provide specific signal activation and osteogenic differentiation of MSCs. However, the expression of BMP2 showed opposite results, where a significant high expression of BMP2 was observed in LC, which indicates that possibly alternative signaling pathways are involved in osteogenic differentiation of cells in different size concavities. Additionally, it is known that the BMP signaling is not a crucial pathway for osteogenic differentiation of AT-MSCs [34, 35]. Other osteogenic genes did not show significant differences between the groups. This observation can be explained by the fact that all experimental samples were obtained from cultures in osteogenic medium containing 5% PL, which promotes osteogenic signaling.

In conclusion, CaP disks covered with different surface concavities (in size) were able to direct MSCs behavior in terms of proliferation and osteogenic differentiation. The results of this study suggest a correlation between concavity size, cell proliferation, cellular 3D organization and osteogenic differentiation of cells within the concavities. Further, the effect of a concavity on the behavior of MSCs will disappear above a certain dimension threshold (i.e. between MC and LC, corresponding to concavity diameter of 800 and 1800 μm, respectively); beyond this threshold, MSCs do not recognize the hemispherical volume in which they are cultured.

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CHAPTER 7

Summary, Closing Remarks and Future Perspectives

Due to the ageing of the world population, healthcare professionals are continuously searching for new or improved treatment approaches for a large variety of medical problems, like e.g. genetic, developmental, and traumatic skeletal disorders [1]. The challenge in the treatment of bone defects is to find a technology that can replace the use of autologous bone grafts. In this context, tissue engineering and regenerative medicine, which both aim to replace damaged tissues, have raised major expectations [2]. Tissue engineering strategies combine the knowledge of (stem) cell biology and biomaterial development. The promise to use stem cells for tissue regeneration is appealing, although the exact way to implement stem cells toward tissue regeneration remains unclear. Characterization of adult stem cells in the form of mesenchymal stromal cells (MSCs) or pericytes [3], isolated from different tissues, e.g. bone marrow and fat tissue, showed promising future for bone tissue engineering and regenerative medicine, but still the actual progression of MSC-based therapies toward clinical application remains at a slow pace [4]. It is anticipated that by guiding the activation of adequate osteogenic signals in these stem cells, upon loading in a properly designed biomaterial

scaffold, an effective contribution to bone regeneration becomes feasible [5].

This thesis aimed to evaluate specific cellular and molecular aspects of cell-cell and cell-biomaterial interactions for tissue engineering applications. In more detail, the research described in this thesis focused on the effect of MSC origin, nutritional additives to the culture medium, properties of biomaterial scaffolds, and co-culture with angiogenic or hematopoietic cells on the behavior and osteogenic differentiation of MSCs. Following specific subaims, the main findings of the research efforts are summarized below.

Chapter 1 provides a brief introduction on bone tissue regeneration strategies, especially cell-cell and cell-biomaterial based strategies, their clinical need and scientific achievements so far. Each following chapter discusses a separate study. This summary addresses the aim described in the first chapter in successive order:

Subaim 1 (Chapter 2): Which signaling pathways are involved in osteogenesis and how can the knowledge on these signaling pathways be applied to improve the success of bone regenerative medicine?

Chapter 2 describes a review of the scientific literature to pinpoint the different signaling pathways involved in osteogenic differentiation of MSCs/ osteogenesis and their potential to be used in bone regenerative treatment. MSCs play a crucial role in natural bone regeneration and remodeling processes, during which their differentiation is regulated by specific signaling pathways. The understanding of this molecular machinery will be a crucial point for the development of bone regenerative treatments, as differences in culture protocols, biomaterial scaffold properties, and interactions with resident cells will lead to changes in MSC behavior, osteogenic differentiation, and hence their contribution to bone regenerative processes. Detailed knowledge about signaling mechanisms, will help to regulate the osteogenic signals in MSCs, which will provide controlled mechanisms, for the designing cell-based tissue engineering constructs. Fundamental screening of osteogenesis-related

complete gene profiles could help to increase the quality of newly designed biomaterials with superior performance in terms of osteoinductivity and osteogenicity. Fundamental knowledge about cell signaling mechanisms can be effectively implemented in bone regenerative treatments, by incorporating signaling molecules via loading or via genetic modification of stem cells, which will help to control cell-cell and cell-biomaterial interactions.

Subaim 2 (Chapter 3): To what extent do cell culture nutritional supplements affect osteogenic differentiation of human BM-MSCs and AT-MSCs? Based on the fact that cell culture nutritional supplements affect the in vitro mineralization of MSCs [6], it become important to analyze the activation of signaling pathways that promote the osteogenic differentiation of BM-MSCs and AT-MSCs.

Chapter 3 we evaluated osteogenic mechanisms in BM-MSCs and AT-MSCs, cultured with different nutritional supplements (i.e. platelet lysate [PL] and fetal bovine serum [FBS]). The osteogenic differentiation and related gene expression profile of common osteogenic genes in different MSCs (i.e. BM-MSCs and AT-MSCs) were the major focus of this study.

In view of mineralization, BM-MSCs showed a significantly higher calcium deposition when cultured in FBS- compared to PL-supplemented media, while AT-MSCs showed opposite results of mineralization i.e. high level of mineralization in PL supplemented media. Gene expression results showed that BM-MSCs in PL-supplemented medium highly express BMP2, BMP4, Wnt1, Runx2, and osterix in contrast to AT-MSCs, which only highly expressed Runx2. Additionally, a high level of parallelism in BMP2, BMP4 and Wnt1 gene expression was observed, irrespective of MSC origin or nutritional supplement. The results of this study showed that (i) the osteogenic gene expression profile is dependent on the origin of the MSCs and nutritional supplements (i.e. FBS or PL), which differently affect cell signaling pathways and related osteogenic differentiation, and (ii) AT-MSCs have high potential

as an alternative to BM-MSCs in application of subsequent (pre-)clinical research, because of their rapid proliferation and high mineralization capacity, especially in PL supplemented media.

Subaim 3 (Chapter 4): How does co-culture of human AT-MSCs with endothelial or hematopoietic cells affect osteogenic signaling and differentiation?

Cell-based bone regeneration is generally pursued based on single cell type approaches, for which MSCs are most frequently used. Because multiple cell types are involved in physiological bone regeneration, including those related to blood vessel formation and inflammation,

Chapter 4 we evaluated the osteogenic differentiation of AT-MSCs upon co-culture with endothelial cells or macrophages in a direct or indirect in vitro co-culture set-up. We assumed that (i) endothelial cells and macrophages would stimulate AT-MSCs proliferation and osteogenic differentiation, and that (ii) these two cell types will more profoundly affect osteogenic differentiation of AT-MSCs in a direct compared to an indirect co-culture set-up, because of the possibility for both cell-cell interactions and effects of secreted soluble factors. The results of this study showed that the osteogenic differentiation of AT-MSCs was indeed stimulated by endothelial cells, particularly in the direct co-culture set-up. Although initial numbers of AT-MSCs in co-culture with endothelial cells were 50% compared to monoculture controls, equal levels of mineralization were achieved. In contrast to endothelial cells, macrophages showed a variable effect on AT-MSCs behavior for indirect co-cultures and a negative effect on osteogenic differentiation of AT-MSCs for direct co-cultures, the latter most likely related to the difference in species from which the cells derived. The results of this study demonstrated potential for the use of cell-cell combination strategies in bone regenerative therapies involving cell-based concepts.

Subaim 4 (Chapter 5): To what extent does the amount of HA nano-crystals

in a composite nHA/collagen hydrogel system affect the osteogenic differentiation of different MSCs?

Hydrogels represent a versatile group of biomaterials with promising properties for bone regenerative research and therapy. Natural hydrogels (i.e. collagen, fibrin) have the advantage of being non antigenic and having intrinsic cellular interaction capacities. Toward biomimetic scaffold systems.

Chapter 5 is focused on the combination of collagen (COL) hydrogels with nano-sized hydroxyapatite (nHA; $Ca_{10}(PO_4)_6(OH)_2$), to comparatively evaluate the *in vitro* effect of biomimetic nHA/COL based composite hydrogels (with different ratios of nHA and COL) on the osteogenic differentiation of human mesenchymal stromal cells (MSCs), isolated from either adipose tissue (AT-MSCs) or bone marrow (BM-MSCs). We hypothesized that (i) nHA/COL composite hydrogels will promote the osteogenic differentiation of MSCs in an nHA concentration dependent manner, and that (ii) AT-MSCs will show higher osteogenic potential compared to BM-MSCs, because of their intrinsic higher proliferation and osteogenic differentiation potential in 2D *in vitro* cultures. The results indicated that AT-MSCs have high proliferation, differentiation and mineralization capacities in nHA/COL constructs, irrespective of nHA concentration, whereas BM-MSCs only showed marginal cell proliferation and osteogenic differentiation in all experimental nHA/COL constructs. Based on the results of ALP activity and OCN protein level, the osteogenic differentiation of BM-MSCs started in the beginning of the culture period and for AT-MSCs at the end of the culture period. At a molecular level, both cell types showed high expression of osteogenic markers (BMP2, RUNX2, OCN or COL1) in both an nHA concentration and time dependent manner. In conclusion, AT-MSCs demonstrated higher osteogenic potential in nHA/COL based 3D micro-environments compared to BM-MSCs, in which proliferation and osteogenic differentiation were highly promoted in a time dependent manner, irrespective of nHA amount in the constructs. The fact that AT-MSCs showed high proliferation and mineralization is appealing for

their application in future pre-clinical research as an alternative cell source for BM-MSCs.

Subaim 5 (Chapter 6): Do surface features of CaP-based ceramics affect cellular organization and osteogenic differentiation of AT-MSCs?

With improved osteogenic capacity of AT-MSCs in HA/collagen based 3D scaffolds (previous chapter)

Chapter 6 is focused on the evaluation of AT-MSC behavior on CaP ceramic scaffolds either or not endowed with micro-scale surface features. In brief, AT-MSCs were cultured on CaP disks featuring hemispherical concavities of various sizes (i.e. 440, 800 or 1800 μm diameter). It was hypothesized that (i) surface concavities would promote cell proliferation, cellular organization within the concavities, and osteogenic differentiation, as a result of a more pronounced 3D micro-environment and CaP nucleation in concavities. MSC (ii) proliferation and osteogenic differentiation would increase with smaller concavity size due to more rapidly occurring 3D cell-cell interactions. We found that indeed, concavity size affects cell proliferation, i.e. 440 μm concavities significantly increased cell proliferation in comparison to planar surface and to 800 and 1800 μm concavities. Additionally, concavity size demonstrated to influence 3D cellular organization within the concavity volume, because already after three days cells, in different size concavities, were distributed differently, i.e. 3D-like manner in SC and MC, and 2D monolayer on the in LC. This means that cells can recognize geometrical properties of the surface and respond differently to their cellular organization. Next, concavity size promoted osteogenic differentiation of cells, as evidenced by increased osteocalcin gene expression in 440 μm concavities, and exclusive osteocalcin staining for 440 and 800 μm concavities, but not for 1800 μm concavities and planar surface controls.

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Closing remarks and future perspectives

The research described in this thesis aimed to study cellular and molecular aspects of the natural bone regeneration process and to create new concepts in cell-based bone tissue engineering strategies for future application in bone regenerative medicine. To achieve this goal, cellular and molecular aspects of MSC osteogenic potential were analyzed.

Molecular analysis of MSCs from different origin (i.e. bone marrow and adipose tissue), showed that the osteogenic gene expression profile is dependent on the origin of MSCs and nutritional supplements, which can differently affect signaling pathways and the subsequent cell proliferation and osteogenic differentiation. This phenomenon of maximized mineralization capacity of AT-MSCs in PL-supplemented and BM-MSCs in FBS-supplemented media still remains elusive. Rapid proliferation and high mineralization activity of AT-MSCs, especially in PL supplemented media, seems appealing for their use in subsequent (pre-)clinical applications. AT-MSCs in the presence of endothelial cells showed a positive effect in terms of osteogenic differentiation and mineralization, which means that endothelial cells apparently interact with AT-MSCs and promote their osteogenic differentiation via cellular crosstalk and exchange of signaling molecules [7, 8]. The stimulated osteogenic differentiation and mineralization of AT-MSCs makes co-culture systems attractive for future (pre-)clinical work. The next step in the search to the potential of co-culture systems will be the investigation of cell behavior in 3D scaffold systems, where cellular interaction can be analyzed in the presence of multiple cell types.

Further, our studies showed that AT-MSCs demonstrated enhanced osteogenic potential in nano-HA/collagen based 3D hydrogel constructs compared to BM-MSCs. Th observation fact that AT-MSCs demonstrated a higher proliferation and mineralization profile can be used as an alternative cell source for BM-MSCs. In addition, the studies indicated that this effect can be

increased further by introducing concavities in the scaffold surface. A correlation was found between concavity size, cell proliferation, cellular 3D organization and osteogenic differentiation. Evidently, AT-MSCs are able to recognize the geometrical and chemical properties of the surface and respond to these geometrical and chemical stimuli [9, 10].

In summary, current strategies in cell-based bone regenerative research are not ideal yet, but tissue engineering approaches hold promise to achieve this goal. Advanced bone tissue engineering requires consideration of many crucial factors, including cellular behavior, cell-cell interaction, material properties, their proper interaction and controlled intracellular signals. Complete screening of the molecular mechanisms (e.g. via microarray and RNA sequencing), controlling cellular behavior and osteogenic differentiation of MSCs will provide a closer look into their molecular machinery. This will enable external control over cellular behavior (into osteogenic lineages) via environmental changes, such as topography, 3D cellular scaffolding or influence with other cell types. However, after each change of external stimuli, cellular behavior needs to be analyzed at the molecular level to understand possible changes in osteogenic signaling pathways. With controllable cellular behavior, it would be possible to modify or develop new engineered tissue constructs (using collagen in combination with other macro-molecules, HA nano-crystals, inorganic elements which are present in bone), which can more closely mimic the bone micro-environment. To analyze cell-cell, cell-biomaterial 3D interaction in the same construct, different cell types, as co-culture system, can be encapsulated in new engineered construct. However, these in vitro observations do not warrant successful translation into (pre-)clinical work. The biological response to new engineered constructs at the cellular as well as molecular level need to be analyzed an in vivo level, in order to understand and to control osteogenic signals in the living organism, where many factors (such as blood supply, interaction of different cell types, immunological response) are influential. Better understanding of *in vivo* biological response to new engineered constructs will help to improve

their structure for future (pre-)clinical applications.

7.1 Samenvatting, Eindopmerkingen en Toekomstperspectieven

Als een gevolg van de steeds ouder wordende populatie, zijn gezondheidszorg professionals steeds op zoek naar nieuwe of verbeterde behandelingsmethoden om een variteit aan medische problemen aan te pakken. Een voorbeeld daarvan zijn de genetische botziekten, de botziekten die ontstaan tijdens de ontwikkeling en de traumatische botproblematiek [1]. The uitdaging in de behandeling van botdefecten is het vinden van een technologie die het gebruik van autoloog bot kan vervangen. In deze context, hebben specialismen binnen de gebieden van weefselontwikkeling en regeneratieve geneeskunde, welke beiden als doel hebben om beschadigd weefsel te vervangen, hoge verwachtingen geschapen [2]. Weefselontwikkelingsstrategien combineren de kennis van (stam)celbiologie en de ontwikkeling van biomaterialen. De belofte om stamcellen te gebruiken binnen de weefselregeneratieve geneeskunde is aantrekkelijk, echter blijft de precieze aanpak om stamcellen te implementeren in weefselregeneratieve methoden onduidelijk. Karakterisatie van volwassen stamcellen in de vorm van mesenchymale stromale cellen (MSCs) of pericyten [3], gesoleerd uit verschillende weefsels, bijvoorbeeld beenmerg en vetweefsel, toonden verwachtingsvolle toekomstperspectieven op het gebied van botweefselontwikkeling en binnen de regeneratieve geneeskunde. Echter, tot op heden gaat de progressie van op MSCs gebaseerde therapien tot aan klinische toepassing nog langzaam [4]. Verwacht wordt, dat doormiddel van het begeleiden van de activatie van adequate osteogene signalen in deze stamcellen, en het combineren van deze stamcellen met een gedegen ontwikkeld biomaterial, een effectieve bijdrage kan worden geleverd aan de botregeneratieve geneeskunde [5] . Dit proefschrift heeft als doel het evalueren van

specifieke cellulaire en moleculaire aspecten van cel-cel en cel-biomateriaal interacties voor toepassing in de ontwikkeling van weefsel. In detail ligt de focus van de onderzoeken binnen dit proefschrift op het effect van MSC afkomst, toevoeging van voedingsstoffen aan het celkweek medium, eigenschappen van de biomaterialen en co-kweek met angiogene of hematopotische cellen op het gedrag en de osteogene differentiatie van MSCs. Op basis van de specifieke doelen van dit proefschrift worden hieronder de belangrijkste resultaten per hoofdstuk samengevat:

Hoofdstuk 1 geeft een korte introductie ten aanzien van botweefsel regeneratieve strategien, voornamelijk cel-cel en cel-biomateriaal strategien, hun klinische relevantie en de wetenschappelijke prestaties op dit gebied tot op heden. Elk volgend hoofdstuk bespreekt een afzonderlijke studie. Deze samenvatting richt zich met opeenvolgende deeldoelen tot het uiteindelijke hoofddoel beschreven in het eerste hoofdstuk:
Deeldoel 1 (Hoofdstuk 2): Welke signalerings routes zijn betrokken bij de osteogenese en hoe kan de kennis van deze signalerings routes toegepast worden om het succes van botregeneratieve geneeskunde te verbeteren?

Hoofdstuk 2 geeft een review op basis van de wetenschappelijke literatuur betreffende de verschillende signalerings routes die betrokken zijn bij osteogene differentiatie van MSCs/osteogenese en hun potentie om gebruikt te worden in botregeneratieve behandelingen. MSCs spelen een cruciale rol in de natuurlijke botregeneratie en in het remodelleringsproces, waarbij gedurende dit proces hun differentiatie wordt gereguleerd door specifieke signalerings routes. Begrip van deze moleculaire mechanismen zal een cruciaal punt zijn in de ontwikkeling van botregeneratieve behandelingen. Verschillen in celkweekprotocollen, eigenschappen van biomaterialen en interacties met residente cellen zal leiden tot veranderingen in het gedrag van MSCs, osteogene differentiatie, en als een resultaat in hun bijdrage aan het botregeneratieve proces. Gedetailleerde kennis van signaleringsmechanismen, zal bijdragen aan het reguleren van osteogene signalen in MSCs, wat zal leiden to gecon-

troleerde mechanismen, voor het ontwikkelen van op cellen gebaseerde weefselconstructies. Fundamentele screening van osteogenese-gerelateerde, complete genprofielen kunnen bijdragen aan een toename van de kwaliteit van nieuw ontwikkelde biomaterialen met een superieure prestatie op het gebied van osteoinductiviteit en osteogenese. Fundamentele kennis van celsignaleringsmechanismen kunnen effectief gemplementeerd worden in botregeneratieve behandelingen, door middel van het incorporeren van signaalmoleculen via lading of via genetische modificatie van stamcellen, wat zal bijdragen aan het cotroleren van cel-cel en cel-biomateriaal interacties.

Deeldoel 2 (Hoofdstuk 3): In hoeverre benvloeden de voedingssuplementen in celkweken de osteogene differentiatie van humane BM-MSC's en AT-MSC's? Uitgaande van het feit dat voedingssuplementen in celkweken de in vitro mineralizatie van MCS's benvloeden, is het belangrijk geworden de activatie van signaleringsmechanismen die osteogene differentiatie van BM-MSC's en AT-MSC's bevorderen, te analyseren.

In **Hoofdstuk 3** is de osteogene differentiatie van BM-MSC's en AT-MSC's, gecultiveerd met verschillende voedingssupplementen (d.w.z. plaatjes lysaat [PL] en foetaal serum van bovine origine [FBS]) gevalueerd. De osteogene differentiatie en gerelateerde genexpressie profielen van de veelvoorkomende osteogene genen in de verschillende MSC's (d.w.z. BM-MSC's en AT-MSC's) waren hoofdfocus van deze studie. Met betrekking tot de mineralizatie, toonden BM-MSCs een significant hogere calciumdepositie wanneer gekweekt in FBS- in vergelijking tot PL-media, terwijl AT-MSC's tegenovergestelde resultaten lieten zien, d.w.z. een hoger niveau van mineralisatie in de PL-gesupplementeerde media. Resultaten van onderzoek naar genexpressie toonden dat BM-MSC's in PL-gesupplementeerde media een hoge expressie hebben van BMP2, BMP4, Wnt1, Runx2 en osterix in tegenstelling tot AT-MSC's, welke alleen een hoge expressie lieten zien van Runx2. Bovendien, werd een hoog niveau van parallelisme gezien van BMP2, BMP4 en Wnt1 genex-

pressie, onafhankelijk van de origine van de MSC of van het voedingssupplement. De resultaten van deze studie laten zien dat (i) het osteogene expressie profiel afhankelijk is van de origine van de MSC's en de voedingssupplementen (d.w.z. FBS of PL), welke de cel signaleringsroutes en de gerelateerde osteogene differentiatie op een verschillende manier benvloeden, en (ii) dat er een hoge verwachting is van AT-MSC's als alternatief voor BM-MSCs wanneer toegepast in (pre-)klinisch onderzoek, vanwege de snelle proliferatie en hoge mineralisatie capaciteit van AT-MSC's, met name in de PL-gesupplementeerde media.

Deeldoel 3 (Hoofdstuk 4): Hoe benvloedt co-celkweek van humane AT-MSC's met endotheelcellen of hematopotische stamcellen de osteogene signalering en differentiatie?

Op cellen gebaseerde botregeneratie is in het algemeen gebaseerd op toepassingen met enkele cell types, waarbij MSC's het meest gebruikt worden. Omdat echter meerdere cel types betrokken zijn bij de fysiologische botregeneratie, waaronder ook de cel types gerelateerd aan vaatformatie en inflammatie, werd in **Hoofdstuk 4** de osteogene differentiatie van AT-MSC's in co-celkweek met endotheelcellen of macrofagen in een directe of indirecte in vitro co-celkweek opzet gevalueerd. We veronderstelden dat (i) endotheelcellen en macrofagen de proliferatie en osteogene differentiatie van AT-MSC's zouden stimuleren, en dat (ii) deze twee cel types duidelijker de osteogene differentiatie zouden benvloeden in een directe co-celkweek opzet dan in een indirecte co-celkweek opzet, vanwege de mogelijkheid voor zowel cel-cel interacties als effecten op de gesecreteerde oplosfactoren. De resultaten van deze studie tonen aan dat de osteogene differentiatie van AT-MSC's inderdaad werd gestimuleerd door endotheelcellen, met name in de directe co-celkweek opzet. Ondanks dat het initile aantal AT-MSC's in de co-celkweek met endotheelcellen 50

Deeldoel 4 (Hoofdstuk 5): In hoeverre benvloedt de hoeveelheid HA nano-

kristallen in een nHA-collageen hydrogel systeem de osteogene differentiatie van de verschillende MSC's?

Hydrogels vertegenwoordigen een veelzijdige groep van biomaterialen met veelbelovende capaciteiten voor onderzoek naar botregeneratie en therapie. Natuurlijke hydrogels (d.w.z. collageen, fibrine) hebben het voordeel dat ze niet antigeen zijn en intrinsieke cellulaire interactie capaciteiten hebben. Ten aanzien van biomimetische scaffold systemen, focust **Hoofdstuk 5** op de combinatie van collageen (COL) hydrogels met nano hydroxyapatiet (nHA; Ca10(PO4)6(OH)2), om het in vitro effect van biomimetische nHA/COL hydrogels (met verschillende ratio's van nHA en COL) op de osteogene differentiatie van humane mesnechymale stromale celeln (MSC's), geisoleerd uit of vetweefsel (AT-MSC's) of beenmerg (BM-MSCs) vergelijkend te kunnen evalueren. De hypothese was dat (i) nHA/COL hydrogels de osteogene differentiatie van MSC's zouden promoten afhankelijk van de nHA concentratie, en dat (ii) AT-MSC's een hogere osteogene potentie zouden laten zien in vergelijking tot BM-MSC's, vanwege de hogere intrinsieke proliferatie en osteogene differentiatie potentie in 2D in vitro celkweken. De resultaten indiceerden dat AT-MSC's over hogere proliferatie, differentiatie en mineralisatie capaciteiten beschikken in nHA/COL constructies, onfahankelijk van de nHA concentratie, terwijl BM-MSC's alleen een marginale cel proliferatie en osteogen differentiatie lieten zien in alle experimentele nHA/COL constructies. Gebaseerd op de resultaten van ALP activiteit en OCN eiwitniveua, begon de osteogene differentiatie van BM-MSC's in het begin van de celkweek periode en voor AT-MSC's aan het einde van de celkweek periode. Op moleculair niveau toonden beide cel types een hoge expressie osteogene markers (BMP2, RUNX2, OCN of COL1), zowel afhankelijk van de nHA concentratie als tijdsafhankelijk. Concluderend laten AT-MSC's een hogere osteogene potentie zien in op nHA/COL gebaseerde 3D micro-omgevingen in vergelijking tot BM-MSC's, waarbij proliferatie en osteogene differentiatie zeer bevorderd werden door de tijd, onafhankelijk van de hoeveelheid nHA in de constructies. Het feit dat AT-MSC's een hoge proliferatie en mineral-

isatie laten zien maakt AT-MSC's interessant voor toepassing in toekomstig pre-klinisch onderzoek als een alternatieve cel bron voor BM-MSC's.

Deeldoel 5 (Hoofdstuk 6): Beinvloeden oppervlakte eigenschappen van CaP keramieken de cellulaire organisatie en osteogene differentiatie van AT-MSC's?

Hoofdstuk 6: Met het oog op de verbeterde osteogene capaciteit van AT-MSC's in HA /collageen 3D scaffolds (voorgaand hoofdstuk), focust hoofdstuk 6 op de evaluatie van het gedrag van AT-MSC's op CaP keramieken scaffolds met of zonder micro-geschaalde oppervlakte eigenschappen. In het kort: AT-MSC's werden gekweekt op CaP schijfjes met verschillende grootte hemisferische concaviteiten (d.w.z. 440, 800 of 1800 m in diameter). De hypothese luidde dat (i) oppervlake concaviteiten de cel proliferatie, cellulaire organisatie in de concaviteiten en osteogene differentiatie zouden bevorderen, als een resultaat van een meer geprononceerde 3D micro-omgeving en CaP nucleatie in de concaviteiten en dat (ii) met kleinere concaviteiten de MSC proliferatie en osteogene differentiatie zouden toenemen ten gevolge van snellere 3D cel-cel interacties. We vonden inderdaad dat de grootte van de concaviteiten de cel proliferatie beinvloedt, d.w.z. concaviteiten met een grootte van 440 m verhoogden significant de cel proliferatie in vergelijking tot vlakke oppervlakten en in vergelijking tot concaviteiten met een grootte van 800 en 1800 m. Ook werd aangetoond dat de grootte van de concaviteiten de 3D cellulaire organizatie in het concaviteiten volume benvloedde, omdat al na drie dagen, cellen in verschllende grootte concavitetien, verschillend waren gedistribueerd, d.w.z. op een 3D manier op SC en MC en in een 2D monolaag op LC. Dit betekent dat cellen geometrische eigenschappen op de oppervlakte kunnen herkennen en verschillend kunnen reageren ten aanzien van hun cellulaire organizatie. Ten tweede werd aangetoond dat de grootte van de concaviteiten de osteogene differentiatie van cellen beinvloedt, als bewezen door een verhoogde genexpressie van osteocalcine bij de 440 m concaviteiten en exclusieve osteocaline kleuring bij de 440 en 800 m concaviteiten, maar

niet bij de 1800 m concaviteiten en de vlakke oppervlakten.

Eindopmerkingen en toekomstperspectieven Het onderzoek beschreven in dit proefschrift doelde op het bestuderen van cellulaire en moleculaire aspecten van de natuurlijke botregeneratie processen en om nieuwe concepten te creren op het gebied van op cellen gebaseerde botweefsel ontwikkelingsstrategien voor toekomstige toepassing in de botregeneratieve geneeskunde. Om dit doel te bereiken, werden de cellulaire en moleculaire aspecten van de osteogene capaciteit van MSC's geanalyseerd. Moleculaire analayse van MSC's van verschillende origine (d.w.z. beenmerg en vetweefsel), toonde dat het osteogene genexpressie profiel afhankelijk is van de afkomst van de MSC's en van de voedinssupplementen, welke op een verschillende manier de signaleringsroutes en opeenvolgend de celproliferatie en osteogene differentiatie kunnen benvloeden. Dit fenomeen van gemaximaliseerde mineralisatie capaciteit van AT-MSCs in PL-gesupplementeerde en BM-MSC's in FBS-gesupplementeerde media blijft tot op heden onvatbaar. Snelle proliferatie en hoge mineralisatie activiteit van AT-MSC's, met name in PL- gesupplementeerde media, lijkt aantrekkelijk voor gebruik in opeenvolgende (pre-) klinische toepassingen. In de aanwezigheid van endotheelcellen laten AT-MSC's een positief effect zien ten aanzien van osteogene differnentiatie en mineralisatie, wat betekent dat endotheelcellen blijkbaar interactie vertonen met AT-MSC's en hun osteogene differentiatie bevorderen middels cellulaire cross-talk en middels uitwisseling van signaleringsmoleculren [1, 2]. De stimulatie van de osteogene differentiatie en mineralisatie van AT-MSC's zorgt ervoor de co-celkweek systemen aantrekkelijk zijn voor toepassing in toekomstig (pre-)klinische werk. De volgende stap in de zoektocht naar de potentie van co-celkweek systemen zal zijn het onderzoeken van gedrag van cellen in 3D scaffold systemen, waarbij de cellulaire interactie kan worden geanalyseerd in de aanwezigheid van meerdere cel types.

Verder toonden onze studies dat AT-MSC's een verbeterede osteogene capaciteit hebben in op nano-HA/collageen gebaseerde 3D hydrogels, in vergeli-

jking tot BM-MSC's. De observatie dat AT-MSC's een hoger proliferatie en mineralisatie profiel laten zien, betekent dat zij gebruikt kunnen worden als alternatieve cel bron voor BM-MSC's. Daarbij indiceren deze studies dat dit effect verder kan toenemen door het toevoegen van concaviteiten aan de oppervlakte van de scaffold. Een correlatie werd gevonden tussen de grootte van de concaviteiten, celproliferatie, cellulaire 3D organisatie en osteogene differentiatie. Klaarblijkelijk zijn AT-MSC's in staat de geometrische en chemische eigenschappen van de oppervlakte te herkennen en te reageren op deze geometrische en chemische stimuli [1, 2]. Samenvattend zijn de huidige strategien in op cellen gebaseerde botregeneratief onderzoek nog niet ideaal, maar zijn weefselontwikkeling benaderingswijzen veelbelovend om dit doel te bereiken. Vooruitstrevende botweefselontwikkeling vereist het in ogenschouw nemen van vele cruciale factoren, inclusief cellulair gedrag, cel-cel interactie, materiaal eigenschappen, hun gepaste interactie en gecontroleerde intracellulaire signalen. Complete screening van moleculaire mechanismen (bijv. via microarray en RNA sequencing), het controleren van cellulair gedrag en osteogene differentiatie van MSC's zal een beter beeld geven van de moleculaire machine. Dit zal controle van cellulair gedrag via veranderingen van de omgeving, zoals topgrafie, 3D cellulaire scaffolding? of invloed van andere celtypes, mogelijk maken. Echter, na elke verandering van externe stimuli zal cellulair gedrag geanalyseerd moeten worden op moleculair niveau, om mogelijke veranderingen in osteogene signaleringsroutes te begrijpen. Wanneer controleerbaar cellulair gedrag mogelijk is, is het ook mogelijk om weefselconstructies (waarbij collageen gebruikt wordt in combinatie met ander macro-moleculen, HA nano-kristallen, inorganische elementen die aanwezig zijn in bot) aan te passen of the ontwikkelen die de micro-omgeving van bot beter kunnen imiteren. Om cel-cel, cel-biomateriaal en 3D interactie in dezelfde constructie te kunnen analyseren, kunnen veschillende celtypes, als co-celkweek systeem, ingegoten worden in een nieuw ontwikkelde constructie. Echter, deze in vitro observaties garanderen geen succesvolle translatie tot (pre-)klinisch werk. De biologische reactie op nieuw ontwikkelde construc-

ties op zowel cellulair als moleculair niveau dient te worden geanalyseerd in vivo, om osteogene signalen in een levend organisme te kunnen begrijpen en controleren, waarbij vele factoren (zoals bloedvoorziening, interactie van verschillende cel types, immunologische reactie) van invloed zijn. Een beter begrip van de in vivo biologische reactie op nieuw ontwikkelde constructies zal bijdragen aan het verbeteren van hun structuur voor toekomstige (pre-) klinische toepassingen.

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Astghik Hayrapetyan

Curiculum Vitae

Astghik Hayrapetyan was born on January 27, 1980 in Yeghegnadzor Armenia. In 1997 she was enrolled in the faculty of Dentistry (Stomatology) at Yerevan State Medical University Armenia, where she obtained her bachelor and master degree in Dentistry. During her study in 2001 she attended the OEAD (Oesterreicher Austauschdienst) scholarship program and spend 4 month in the department of Oral Surgery and Implantology, at Karl Franz University of Graz, Austria. After the graduation (2003), in 2004 she was attended the KAAD (Katholischer Akademischer Auslaender-Dienst) scholarship program and worked as a guest researcher in the department of Periodontology at University Dental Clinic of Heidelberg, Germany. In 2008 she started the second Master in Bimolecular Science at Vrije University of Amsterdam, the Netherlands. In November 2011 she

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Publication list

1. Signaling pathways involved in osteogenesis and their application for bone regenerative medicine Astghik Hayrapetyan, John A Jansen, Jeroen JJP van den Beucken Tissue Engineering part B (in press).

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5. Effect of Calcium Phosphate Ceramic Substrate Geometry on Mesenchy-

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6. Controlled Release of Platinum-bisphosphonate Complexes from Injectable Calcium Phosphate Cements for Treatment of Bone Tumors Kambiz Farbod, Kemal Sariibrahimoglu, Alessandra Curci, Astghik Hayrapetyan, Jan N.W. Hakvoort, Jeroen J.J.P. van den Beucken, Michele Iafisco, Nicola Margiotta, Sander C.G. Leeuwenburgh Tissue Engineering Part A (accepted)

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